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Chiara Peila

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Chiara Peila

Promotors

Prof. dr. D. Gazzolo (Ius Promovendi, Alessandria, Italy)
Prof. dr. G.H.A. Visser (Utrecht)
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Content

Abbreviations

- Adrenomedullin (AM)
- Carbon monoxide (CO)
- Central nervous system (CNS)
- Cerebral palsy (CP)
- Cerebrospinal fluid (CSF)
- Coefficient of variability (CV)
- Donor milk (DM)
- Enzyme-Linked Immunosorbent Assays (ELISA)
- Epidermal GF (EGF)
- Epidermal-like GF (HB-EGF)
- Exclusive breastfeeding (EBF)
- Exclusive human milk including expressed and donated breast milk (EHM-ED)
- Extremely low birthweight (ELBW)
- Gestational age (GA)
- Granulocyte-Granulocyte-Colony Stimulating Factor (GCSF)
- Growth factors (GF)
- Heat shock proteins (HSPs)
- Hemeoxygenase-1 (HO-1)
- Heparin-Binding Hepatocyte GF (HGF)
- Holder Pasteurization (HoP)
- Human milk (HM)
- Human Milk bank (HMB)
- Human Milk Banking Associations (HMBA)
- Immunoglobulins (Ig)
- Information-dependent acquisition (IDA)
- Insulin-like GF (IGF)
- Interferon (IFN)
- Interleukin (IL)
- Italian Association of Human Milk Donor Banks (AIBLUD)
- Low birthweight (LBW)
- Macrophage Colony-Stimulating Factor (GM-CSF)
- Macrophage Inflammatory Protein-1β (MIP-1β)
- Mass spectrometry (MS)
- Medium-chain fatty acids (MCFAs)
- Monocyte Chemotactic Protein (MCP)
- Necrotizing enterocolitis (NEC)
- Neonatal intensive care unit (NICU)
- Neurobiomarkers (NB)
- No-Holder pasteurized (NO-HoP)

- Nutritional quality (NQ)
- One-dimensional electrophoresis (1DE)
- Preterm birth (PB)
- Radial Immunodiffusion Assays (RIA)
- Transforming growth factor (TGF)
- Tumor Necrosis Factor (TNF)
- Vermont Oxford Network (VON)
- Very low birthweight (VLBW)
- Weight at birth (BW)

OUTLINE OF THE THESIS

In Chapter 1, part 1, we provide an overview on the several biological aspects of the HM, its current techniques of heat and cold storage and the putative role of emerging neurobiomarkers in HM namely Activin A, S100B, HO-1 and AM.

In Chapter 1, part 2, the state of art on the effect of Holder pasteurization (HoP) on nutrients and biologically-active components in DM is given. Results confirm that HoP affects several HM components and show the difficulties met to quantify the degradation level. Finally, proteins are more significantly affected by HoP but the results concerning specific biologically active molecules remain uncertain.

In Chapter 2 we evaluated the effect of HoP on the protein profile using a semi-quantitative GeLC-MS analysis technique. Results showed that HoP affected about 30% of protein amount in the samples analyzed. The main changes concerned the colostrum, while no evident changes were observed in mature milk. This data offers additional support to the use of DM.

In Chapter 3 we investigated the effects of HoP on the DM concentration of the neurotrophic factor Activin A. The results showed that HoP does not affect Activin A concentrations in DM at different milk maturation degrees. The findings open-up to further investigations on other neuro-biomarkers assessment in HM and their pre-analytical stability according to storage procedures.

Therefore, in Chapter 4 we investigated whether HoP procedure could somehow affect the neurotrophic S100B protein known for its thermos stability properties. Surprisingly, results showed that HoP affected S100B levels in DM, in transitional and mature milks, whereas no differences were detectable in colostrum. The explanation main reside in the HoP procedure itself (heat treatment at 62.5° for 30 minutes) suggesting that the duration of the procedure rather than temperature can cause protein's degradation.

In Chapter 5 we investigated the effects of HoP on a heat shock protein (HSPs) namely HO-1 known to participate in a cascade of events. HO-1 may play a role in development and regulation of the immune system. Our results showed that HoP did not affect HO-1 in DM at different degrees of maturation. The finding is of relevance bearing in mind the role of HO-1 in the development and regulation of the immune system and of the gastro-intestinal tract.

Finally, in Chapter 6 we evaluated the effect of cold storage of HM on the concentrations of a vasoactive and angiogenic factor namely AM. Results showed that AM concentrations in HM are temperature and time-dependent. The present data suggest caution in the storage procedure and duration. The finding is of relevance taking into account the crucial role of the peptide in systemic vascular and multiorgan development

In Chapter 7 we present the summary and conclusions of this thesis.

Chapter 1

Introduction – Part 1

- The preterm infants
- Breastfeeding in the NICUs
- Human Milk and biological aspects
- Pasteurized Donor Human Milk
- Refrigerated Human Milk
- Neurotrophic factors — Activin A
- Calcium binding proteins — S100B
- Oxidative stress biomarkers — HO-1
- Vasoactive Agents – AM
- Aim of the thesis

The preterm infant

Preterm birth (PB) is commonly defined as birth occurring <37 weeks gestational age (GA) and complicates 5-10% of all birth in Europe. In USA the rate is about 12–13% supporting the notion that PB varies from country to country (Figure1) [1].

PB can be classified according to GA in extreme PB (less than 28 weeks), severe PB (28–31 weeks), moderate PB (32–33 weeks), and late PB (34–36 weeks). PB can be also classified according to weight at birth (BW) as extremely low birthweight (<1000g, ELBW), very low birthweight (1001-1500 g, VLBW) and low birthweight (1501-2500g, LBW) [1].

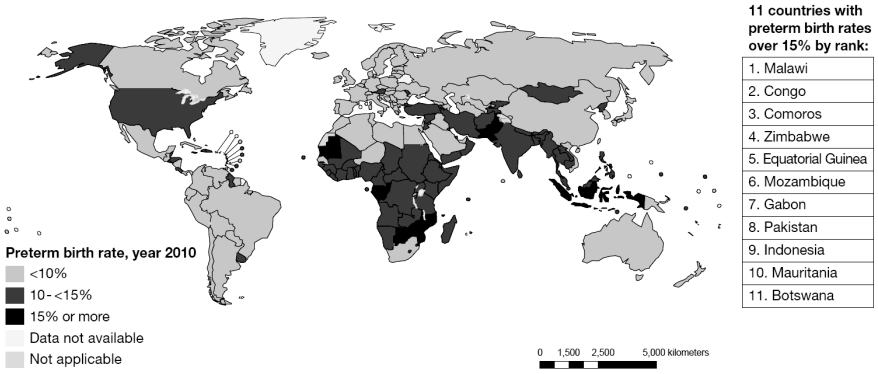

Figure 1. Preterm birth rate in developed and developing countries.

Despite advances in the identification of risk factors and mechanisms related to preterm labor, and the introduction of many public health and medical interventions, the PB rate has risen in most developed countries in recent decades [3,4]. Much of this increase can be explained by rising numbers of medically indicated PB as a consequence of maternal or fetal problems and by rising number of preterm multiple pregnancies associated with assisted reproductive technologies [5,6].

The highest mortality rates occur in VLBW infants [7]. The most common neurodevelopmental complications in the early years of life of PB survivors are cerebral palsy (CP), mental retardation and sensory impairments (such as visual and auditory deficits). The definitions of the disabilities and their severity are not uniform, which makes interpretation difficult, but about 25% of survivors have neurological disabilities [8,9]. Furthermore, these complications are inversely related to GA. There is growing evidence that perinatal therapeutic strategies (i.e. antenatal steroids), careful delivery room management guaranteeing an adequate transitional phase, neonatal intensive care unit (NICU) treatments (respiratory support, surfactant) have resulted in a considerable decrease in perinatal mortality and morbidity. Nonetheless, there is an important role

for promotion of human milk. The latter is included in the indicators of clinical activity in the main neonatal networks (i.e. Vermont Oxford Network, VON) and a cut-off rate of >20% of HM at discharge has been internationally agreed [10].

Breastfeeding in the NICUs

The importance of nutrition in the early period of life is now well known: the term *"programming"* has been proposed to emphasize that early nutrition should be considered both in terms of immediate nutritional needs and for long-lasting/life-long biological effects [11]. The mother her own milk is widely considered as the optimal feeding and also provides health benefits that are of vital importance for preterm infants in NICUs [10]. Feeding HM to preterm infants decreases rates of infection, necrotizing enterocolitis (NEC) and mortality, while improving neurocognitive and cardiovascular outcomes in the long term [10,12].

A recent multicenter Italian study has reported the incidence of breastfeeding and use of HM among NICUs: at discharge 28% of infants were fed exclusively with HM (Table1). The proportion of infants not fed with HM varied from 6% to 82% across different centers [13].

Table 1. Incidence of human milk at discharge from hospitals in Italy in relation to birth weight.

Birth Weight Categories, g	Exclusive HM		Predominant HM	Complementary Feeding	No HM	Total
	EBF	EHM-ED				
< 1500	18 (3.1%)	163 (27.6%)	1 (0.2%)	142 (24.1%)	266 (45.1%)	590 (100.0%)
1500-1999	7 (1.2%)	145 (24.3%)	3 (0.5%)	263 (44.1%)	178 (29.9%)	596 (100.0%)
2000-2499	17 (2.6%)	123 (19.1%)	0 (0.0%)	348 (54.0%)	157 (24.3%)	645 (100.0%)
≥ 2500	126 (11.3%)	239 (21.4%)	4 (0.4%)	494 (44.2%)	254 (22.7%)	1117 (100.0%)
Total	168 (5.7%)	670 (22.7%)	8 (0.3%)	1247 (42.3%)	855 (29.0%)	2948 (100.0%)

Abbreviations: EBF, exclusive breastfeeding; EHM-ED, exclusive human milk including expressed and donated breast milk; HM, human milk.

In this context the crucial role of health care workers, including pediatricians, to protect, promote and support breastfeeding, has to be emphasized. Health care workers should be trained in breastfeeding issues and counselling. Lack of feeding with breast milk is an important and costly problem for preterm infants that, if addressed successfully, has the potential to contribute to overcome inequalities in health [14].

Human Milk and biological aspects

HM can be considered a species-specific biological "dynamic" system. Particular attention should be given to specific bioactive and immunomodulatory factors, such as gastrointestinal hormones, immunoglobulins, lactoferrin, lysozyme, oligosaccharides, nu-

cleotides, growth factors, enzymes, antioxidants and cellular components. They not only ensure adequate host defense against infections, but also actively modulate the immune response, modify the intestinal bacterial flora and are involved in the regulation of optimal newborn growth including that of the brain when compared to formula milks [15-17]. However, there is growing evidence that these compounds, *per se*, cannot be considered the only responsible of these crucial properties: to date, the hypothesis that other molecules of interest, namely *"trophic factors"* could also be involved in these developmental processes is therefore consistent [18-20] These latter include neuro-oxidative stress biomarkers, neurotrophic and calcium binding proteins known to be involved in a cascade of events leading to brain, cardiac and vascular development/damage [18-20]. The importance of these factors is also emphasized by their presence in HM, at up to 20 times or more higher concentrations than in other biological fluids (i.e. cerebrospinal, CSF; blood; amniotic; urine) [19-22].

Selected biomarkers have a special role in human milk, such as: i) Neurotrophic factors — Activin A; ii) Calcium binding proteins — S100B; iii) Oxidative stress biomarkers — hemeoxygenase-1 (HO-1), iv) Vasoactive Agents – Adrenomedullin (AM).

Pasteurized Donor Human Milk

When mother's milk is unavailable or in short supply, donor milk (DM) represents a second best alternative. The American Academy of Pediatrics, in its most recent policy statement on breastfeeding, recommends that pasteurized DM should be used if mother's own milk is unavailable or if its use is contraindicated [10]. To offer this opportunity to preterm infants, HM should be obtained from a HM bank (HMB). The number of HMBs is rapidly increasing worldwide. At present, in Europe, there are 186 HMBs, and new banks will be established with the support of the European Milk Bank Association [23]. In many countries, national policies to improve infant health outcomes consider DM obtained from an HMB to be a reasonable and effective tool in the delivery of health care to infants and children [24]. DM should be obtained from established HMBs that follow specific guidelines for screening, storage, and handling procedures to optimize its composition while ensuring its safety for the recipient. Many countries now have their own HMB guidelines [25,26]. Pasteurization of the milk minimizes the risk of disease transmission, inactivating most of the viral and bacterial contaminants. In addition, donors are screened in a similar way as blood donors. No report has been published showing transfer of diseases through pasteurized DM, although milk may contain microorganisms [27]. Nevertheless, HMBs, like blood banks, should be aware of the threat of emerging (milk transmissible) pathogens that are not included in contemporary screening protocols. Currently, pasteurization, performed at 62.5 °C for 30 min (Holder Pasteurization, HoP), is recommended for this purpose in all international guidelines for establishment of HMBs [26].

Holder Pasteurization is necessary but partially seems to affect the nutritional and immunological properties of breast milk. Other alternative methods to improve the biological quality and safety of DHM are under investigation: high-temperature short-term pasteurization (flash pasteurization, 72°C for 5–15 seconds) and its homemade low-tech variant for developing countries (flash-heat treatment), thermos-ultrasonic treatment, high-pressure processing, and Ohmic heat treatment. The data about safety for microbiological control are still scarce for most of these novel technologies, and consensus on processing conditions is necessary for non-thermal technologies, before any conclusions on the qualitative and nutritional advantages of these techniques can be drawn [28].

Refrigerated Human Milk

Under some circumstances HM extraction and refrigerated storage may be necessary. The storage of refrigerated milk may also take place at hospitals (mainly in NICUs) or at home for milk to be donated to milk banks or for later use by the mother her own infant. The maximum refrigeration time for human milk ranges between 24 hours and 8 days, according the current advices on safe HM storage [29,30]. Such variability reflects the heterogeneity of the scientific sources, which is attributable to differences in study design and in methodological approach [31]. Depending on the length and on the type of storage, HM may lose some important nutritional and functional properties [32-35]. Recent studies have shown that human milk may be stored for 96h at 4°C without affecting the overall milk integrity, as defined by bacterial growth, white cell count, pH, osmolality, and total protein count and protein profile. Moreover, Giribaldi et al. observed that the refrigeration process did not affect the abundance of important nutritional markers such as lysine, sIgA, lactoferrin and lysozyme content [36,37].

Neurotrophic factors — Activin A

Activin A is a dimeric protein belonging to the transforming growth factor beta (TGF-beta) superfamily. It is composed of two beta-A subunits exerting their biological activity through two different receptors and by the expression of the follistatin gene coding for the activin-binding protein follistatin, which inhibits its biological effects [38,39]. Activin A, its receptors, and binding proteins are widely distributed throughout the brain. Studies in experimental models and in humans strongly correlate enhanced Activin A expression as a common response to acute neuronal damage of various origins. Hypoxic/ischemic injury, mechanical irritation, and chemical damage of brain evoke a strong up-regulation of Activin A [40]. Subsequent experimental studies have shown that Activin A has a beneficial role to neuronal recovery by activating different path-

ways, Activin A exerts neuroprotective activities as well as it modulates cellular and tissue growth and differentiation [38]. On the basis of these findings it is reasonable to argue that Activin A for its involvement in embryogenesis and fetal development, could be detectable in biological fluids with a unique trophic effect such as breast milk. In this regard, Luisi et al. [39] investigated whether Activin A and follistatin as putative protective agents in central nervous system (CNS) and cardiac development were indeed present in human milk. Immunohistochemistry showed positivity for Activin A and follistatin in human milk [19]. Of note, Activin A and follistatin concentrations were significantly higher than those detected in conventional biological fluids. The authors concluded that the presence of Activin A and follistatin in breast tissue is related with their hormone/growth effect modulating growth and differentiation of mammary epithelial cells during lactation. Finally the Activin A and follistatin release in the neonatal circulation, suggests their involvement in the regulation of growth/function of various neonatal tissues including brain and cardiac tissues [41,42].

Calcium binding proteins — S100B

The S100B protein (beta–beta dimer) is a member of a multigenic family of calcium-binding proteins (S100 proteins) [43]. At least 21 proteins belong now to the S100 protein family, the members of which exhibit a pair of so-called EF-hand (i.e. helix-loop-helix) calcium-binding motifs, which are able to change the conformation of the protein after binding to calcium. S100B is mainly concentrated in the CNS, where it is mainly located in the glial cells, astrocytes and Schwann cells, and more recently it has been shown in specific neuronal subpopulations [44]. The protein is present extracellularly, intracellularly, and in the cytosol; its half-life is about 1-h, and the 98% of the protein is eliminated by the kidney route [45]. S100B in CNS regulates several cellular functions (cell–cell communication, cell growth, cell structure, energy metabolism, contraction, and intracellular signal transduction). Elevated S100B levels have been found in different biological fluids (i.e. CSF, blood, amniotic fluid, urine and saliva) in brain damaged fetuses and newborns [44,45]. Experimental and clinical data have shown that S100B behaves like a Janus face: at nanomolar (physiological) concentrations it acts as a cytokine with a neurotrophic effect, while at micromolar concentrations it is neurotoxic and participates in a cascade of events leading to cell death and/or apoptosis [44]. On the basis of the present findings the detection of S100B in breast milk, offers additional support to the debate of the protein's involvement in fetal/neonatal brain development. In particular, S100B concentrations in human milk were 200 times higher than in other biological fluids and higher than in other mammalian species (i.e. cow, goat, donkey and sheep) [21,46]. More recently it has been reported that the protein's concentration in milk formulae may be affected by industrial process procedures utilized for milk's preparation [47].

Oxidative stress biomarkers — HO-1

HO-1 is a stress-inducibile rate-limiting enzyme that catalyzes the breakdown of pro-oxidant heme into biliverdin, carbon monoxide (CO) and free iron, which can drive the synthesis of ferritin for iron sequestration [48]. Several studies have recognized HO-1 to be involved in several cytoprotective effects, due to its multiple catalytic by-products. The multifunctional roles of HO-1 have been shown in the vascular system, including vascular tone regulation, anti-smooth muscle proliferation and anti-endothelial apoptosis. More recently, it also has been shown to have a role in angiogenesis [49]. HO-1 is expressed in the human brain and is associated with brain tumors and neurodegenerative diseases. Two genetically distinct isoenzymes have been identified: HO-2, which is constitutively expressed in various tissues and cells, and HO-1/HSP32 which is highly induced by many factors, including heavy metals, endotoxin, cytokines, heme, nitric oxide, hypoxia, and UV irradiation. It is interesting to note that induction of HO-1/HSP32 expression by means of natural compounds contributes to protection against liver damage in various experimental models [50,51]. Both isoforms are present in the endoplasmic reticulum, however, under certain conditions HO-1/HSP32 it has been shown to be able to translocate into other cellular compartments such as nuclei and mitochondria [52,53]. Li Volti et al. [20] investigated the pattern of HO-1/HSP32 protein in human and milk-formula milks by HO-1/HSP32 assessment in milks together with a computational approach. The results showed the presence of HO-1/HSP32 in human milk, in particular, molecular modeling approach might explain a putative binding between HO-1/HSP32 and his specific receptors (CD91) in the extracellular space playing a major role in the immune system regulation [20,54-56].

Vasoactive Agents – AM

AM is a C-amidated peptide belonging to the calcitonin gene-related peptide family [57] first isolated from human pheochromocytoma by its capability to increase cyclic adenosine monophosphate production [58]. AM and its mRNA have been found in many tissues from different species. The main function of AM is vasodilatation [58], although other actions have been reported, including neuromodulation [59] and inhibition of apoptosis [60,61]. Expression of the AM gene is up-regulated by hypoxia [62,63] and inflammation [64], which are associated with neo-vascularization. Studies in animal models revealed that AM plays an important role in vascular formation in embryos [65-67]. In the CNS, AM is generally localized in neurons and the endothelium [68], in particular in the hypothalamus [69] and in the caudate-putamen where it has been found in the neuronal nuclei [70]. This area is one of the most sensitive brain areas to hypoxic damage. In fact, AM is involved in response to hypoxia, at least in part by means of the

transcription of the Hypoxia-Inducible Factor-1, which enhances AM expression and stabilizes AM mRNA [71].

AM has been implicated in the modulation of several physiological functions including cardiovascular tone, central brain activity, bronchodilation, renal function, hormone secretion, cell growth-differentiation and immune response [72-81]. AM has been also assessed for evaluation of beneficial/side-effects of in-utero vasodilation therapeutic strategies in pregnancies complicated by fetal chronic hypoxia [74-76].

Recent studies indicate that AM is also synthesized in the mammary gland and secreted in breast milk, although the data are discordant with respect to its concentration. On the other hand the presence of AM in HM and the variation in its concentration between the different milk maturation degrees suggest that AM may have an important role in the regulation of growth and maturation of the neonatal gastrointestinal tract [82-84].

Aim of the Thesis

There is growing evidence that in HM and DM oxidative stress biomarkers, neurotrophic proteins and calcium binding proteins are involved in a cascade of events leading to brain, cardiac and vascular development/damage. On the basis of the aforementioned findings, the purpose of the present thesis was to investigate the effects of storage on HM constituents. The issues addressed are:

i) the state of art of HoP effects on HM composition;
ii) the changes in HM qualitative protein profile following HoP procedure;
iii) the potential side-effects due to HP procedure on biomarkers such as Activin A, S100B, HO-1;
iv) the potential side-effects due to prolonged refrigerated storage on AM.

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Introduction – Part 2

The effect of Holder pasteurization on nutrients and biologically-active components in donor human milk: A review

CHIARA PEILA[1], GUIDO E. MORO[2], ENRICO BERTINO[1], LAURA CAVALLARIN[3], MARZIA GIRIBALDI[3,4], FRANCESCA GIULIANI[1], FRANCESCO CRESI[1], ALESSANDRA COSCIA[1]

[1]Complex Structure Neonatology Unit, Department of Public Health and Pediatric, University of Turin; [2]Italian Association of Human Milk Banks, Milan, Italy; [3]Institute of Sciences of Food Production, National Research Concil, Italy; [4]Food & Nutrition Research Center, Council for Agricultural Research and Economics, Italy

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Abstract

When a mother's milk is unavailable, the best alternative is donor milk (DM). Milk delivered to Human Milk Banks should be pasteurized in order to inactivate the microbial agents that may be present. Currently, pasteurization, performed at 62.5 °C for 30 min (Holder Pasteurization, HoP), is recommended for this purpose in international guidelines. Several studies have been performed to investigate the effects of HoP on the properties of DM. The present paper has the aim of reviewing the published papers on this topic, and to provide a comparison of the reported variations of biologically-active DM components before and after HoP.

This review was performed by searching the MEDLINE, EMBASE, CINHAL and Cochrane Library databases. Studies that clearly identified the HoP parameters and compared the same DM samples, before and after pasteurization, were focused on.

A total of 44 articles satisfied the above criteria, and were therefore selected. The findings from the literature report variable results. A possible explanation for this may be the heterogeneity of the test protocols that were applied. Moreover, the present review spans more than five decades, and modern pasteurizers may be able to modify the degradation kinetics for heat-sensitive substances, compared to older ones. Overall, the data indicate that HoP affects several milk components, although it is difficult to quantify the degradation degree. However, clinical practices demonstrate that many beneficial properties of DM still persist after HoP.

Key words: human milk; donor milk; holder pasteurization; Human Milk Banks.

Introduction

Human milk (HM) is the gold standard for the feeding and nutrition of preterm and term newborns [1,2,3]. A mother's own milk is the first choice for improving the short- and long-term outcomes for all neonates [1]. However, the benefits of HM are mediated by several specific bioactive and immunomodulatory factors, and HM can be considered a species-specific biological "dynamic" system [4]. When a mother's own milk is unavailable or in short supply (a common occurrence in Neonatal Intensive Care Units), the World Health Organization and the American Academy of Pediatrics recommend the use of donor milk (DM) as the best alternative [1,3]. Milk delivered to Human Milk Banks (HMBs) should be pasteurized so as to inactivate viral and bacterial agents [5]. The ideal pasteurization process should consist of a phase of rapid heating, followed by a phase in which the temperature is maintained constant, and a final phase of rapid cooling.

Currently, a pasteurization process at 62.5 °C for 30 min (the Holder pasteurization method, HoP) is recommended in all international guidelines for the constitution of HMBs [5,6]. Pasteurized milk is known to retain many beneficial and protective effects of HM. This method is thought to lead to a good compromise between the microbiological safety and nutritional/biological quality of DM [7,8,9,10,11].Nonetheless, it is also well known that HoP affects some of the nutritional and biological properties of HM. Several studies have been performed to investigate the effects of Holder pasteurization on the nutritional and immunological properties of DM, but a comprehensive review and comparison of the related results is, to the best of the authors' knowledge, not available in literature. Currently, the available data consist of reviews regarding the advantages of donor milk, but very few details are given on the effects of HoP on the nutrients of mother's milk [12,13,14,15,16].

Thus, the present paper is aimed at reviewing the published papers, and at comparing the results related to the effects of HoP on the biological and nutritive components of DM.

Search Methodology

The literature review was performed by conducting electronic searches of MEDLINE, EMBASE, CINHAL and the Cochrane Library. The electronic search used the following keywords and MeSH terms: (i) donor milk; (ii) banked milk; (iii) milk bank; (iv) milk banking; (v) (human milk OR donor milk) AND Holder pasteurization; (vi) (human milk OR donor milk) AND pasteurization; (vii) (human milk OR donor milk) AND storage; (viii) (human milk OR donor milk) AND heat treatment; (ix) (donor milk OR Holder pasteurization) AND protein; (x) (donor milk OR Holder pasteurization) AND enzyme; (xi) (donor milk OR Holder pasteurization) AND lipid; (xii) (donor milk OR Holder pasteurization)

AND saccharide; (xiii) (donor milk OR Holder pasteurization) AND vitamin; (xiv) (donor milk OR Holder pasteurization) AND minerals; (xv) (donor milk OR Holder pasteurization) AND oxidative stress; (xvi) (donor milk OR Holder pasteurization) AND cytokine; (xvii) (donor milk OR Holder pasteurization) AND hormone; (xviii) (donor milk OR Holder pasteurization) AND growth factor; and (xix) (donor milk OR Holder pasteurization) AND immunoglobulin. No limits concerning the publication date were set.

Considering differences between the research protocols published to date, we focused the review on studies with an experimental design that:

- defined exactly the pasteurization method precisely (62.5–63 °C for 30 min); and
- compared the same samples of HM before and after the heat treatment.

In order to limit bias in the inclusion/exclusion process, the selection was made with the consensus of two authors (CP and MG).

Table 1. Materials and Methods of the different studies included in the survey.

Ref *	Preterm/Term	Phase of Lactation	Expression Method	Status	Pre-Pasteurization Storage	Pasteurization Equipment	Sample Size	Analytical Method °
[17]	N/A	Mature	N/A	Frozen	−20 °C up to 6 months; thawing in a water bath at 37.5 °C	Sterifeed: pre-heated water bath (63.2 °C); 62.5 °C for 30′; cooling in cold water bath	17 pools—4 donors each	Adiponectine: RIA
								Insulin: electrochemiluminescence immunoassay
								Total fat: creamatocrit
								Total protein: BCA
								Total energy: bomb calorimetry
								Glucose: enzymatic method
[18]	Preterm and Term	Mature	Hand or electric/manual pump	Frozen	−20 °C until processing; thawing and heating to 40 °C using a thermostatic bath	62.5 °C for 30′; cooling to <4 °C in stirred thermostatic baths	34 samples—28 donors	Infrared Analyzer (MIRIS)
[19]	N/A	N/A	Hand or electric/manual pump	Fresh	No	62.5 °C for 30′	57 samples	Infrared Analyzer (Milko-scan Minor)
[20]	Preterm and Term	Colostrum	Hand	Fresh	No	62.5 °C for 30′	36 samples: <32 weeks; 32 samples: 32–36 weeks; 33 samples: >36 weeks	Total protein: refraction index
								Lysozyme: lysoplate method
								Immunoglobulins: RIA
[21]	Term	N/A	N/A	Fresh	No	LABU-Muttermilch pasteurizer: 62.5 °C for 30′	4 Samples—2 CMV-positive and 2 CMV-negative donors	Total protein, alkaline phosphatase and lipase activity: Hitachi 917 Automatic Analyzer
								Folic acid, Vitamin B12: chemiluminescence immunoassays
								sIgA and lysozyme: RIA
[22]	N/A	Mature	Electric pump	Fresh	No	VLM exchangeable HBV-Q-16-16: 63 °C for 30′	30 samples—30 donors	Lysine content: fluorimetry
								Total protein: Lowry method

Table 1. *Cont.*

Ref *	Preterm/Term	Phase of Lactation	Expression Method	Status	Pre-Pasteurization Storage	Pasteurization Equipment	Sample Size	Analytical Method ◦
[23]	Term	Mature	Hand or electric/manual pump. Occasional drip milk	Frozen	−20 °C up to 15 days; thawing in a microwave oven	62.5 °C for 30′; cooling by ice-cold water for 10′	15 samples from individual mother or pool (5 donors)	Total fat: crematocrit
								Total protein: Lowry method
								Lactose: picric acid method
								Vitamin A: HPLC
								Zinc: Atomic absorption spectrometry
[24]	N/A	Mature	N/A	Fresh	Refrigeration at 4 °C for 1 to 2 days; centrifugation at 2 °C for 1 h; −30 °C until testing	62.5 °C for 30′	1 pool—25 donors	Immunoglobulins and lactoferrin: RIA
								Vitamins: labeled cyanocobalamin, separation of free and protein-bound vitamins by gel filtration
[25]	N/A	N/A	N/A	Frozen	−40 °C until analysis	62.5 °C for 30′	1 pool—10 donors	Total protein: BCA
								Immunoglobulins: ELISA
								Lysozyme activity: *Micrococcus lysodeikticus* turbidimetric assay
[26]	N/A	Mature	N/A	Fresh	Refrigeration	62.5° C for 30′ in stirred water bath	2 pools—5 and 6 donors	Immunoglobulins: ELISA
[27]	Term	Mature	Electric pump	Fresh	–80 °C until the analysis	62.5 °C for 30′	10 samples—10 donors	Immunoglobulins: ELISA
[28]	N/A	Mature	N/A	Fresh	No	63 °C for 30′	23 samples	Immunoglobulins: RIA
[29]	Term	Colostrum, transitional and mature	Manual or pump	Fresh	Refrigeration in ice	62.5 °C for 30′	5 samples—89 donors	Immunoglobulins: RIA
								Lactoferrin: Laurell method
[30]	N/A	N/A	Overflow milk	Fresh	Refrigeration up to 48 h	62.5 °C for 30′	16 samples	Electroimmunoassay against monospecific antiserum
[31]	N/A	Colostrum and mature	HMB protocol	Fresh	N/A	62.5 °C for 30′	10 colostrum and 8 mature milk	Furosine: HPLC
								Carbohydrates: gas chromatography
								Cytokines: ELISA
								Immunoglobulins: ELISA

Table 1. *Cont.*

Ref *	Preterm/Term	Phase of Lactation	Expression Method	Status	Pre-Pasteurization Storage	Pasteurization Equipment	Sample Size	Analytical Method °
[32]	N/A	Colostrum	Hand or electric pump	Fresh	−20 °C until analysis	62.5 °C for 30′	1 pool—11 donors	Immunoglobulins: ELISA
								Lysozyme activity: *Micrococcus lysodeikticus* turbidimetric assay
								Lactoperoxidase activity: ABTS assay
[33]	Term	Transitional	Electric pump	Fresh	No	Metallarredinox: 62.5 °C for 30′	1 pool—4 donors	IgA, lactoferrin: SDS-PAGE, Western Blot and mass spectrometry
								Lipase activity: p-nitrophenol assay
								Available lysine: OPA method
[34]	N/A	N/A	N/A	Fresh	N/A	3 devices: Sterifeed–Saurin–Carag: 62.5 °C for 30′	10 samples for Sterifeed; 6 samples for Saurin; 6 samples for Carag	Lysozyme, sIgA and lactoferrin: ELISA
[35]	N/A	N/A	N/A	Frozen	−20 °C until analysis	62.5 °C for 30′	10 samples—10 donors	Lysozyme, sIgA and lactoferrin: ELISA
[36]	N/A	N/A	Drip milk	Fresh	No	Semi-automated Holder pasteurizer	1 pool—20 donors	IgA: electroimmunoassay
								Lysozyme activity: *Micrococcus lysodeikticus* turbidimetric assay
[37]	N/A	Mature	Electric pump	Frozen and fresh	–70 °C; thawing in cool water	62.5 °C for 30′ in stirred water bath	6 samples: 3 pools from HMB and 3 samples from 3 donors	Total fat: gravimetry
								Fatty acids: gas chromatography
								Lipase activity: triglyceride emulsion
								Amylase: ELISA
[38]	N/A	Mature	N/A	Frozen	4 °C overnight; thawing at 37 °C in water bath, gently mixed	Sterifeed: pre-heated water bath (63.2 °C); 62.5 °C for 30′; cooling in cold water bath	17 pools—4 donors each	Cytokines and growth factors: ELISA
								Fatty acids: gas chromatography

Table 1. *Cont.*

Ref *	Preterm/Term	Phase of Lactation	Expression Method	Status	Pre-Pasteurization Storage	Pasteurization Equipment	Sample Size	Analytical Method ◦
[39]	N/A	Mature	N/A	Fresh	Refrigeration until analysis	62.5 °C for 30′ in water bath under constant agitation	1 pool—6 donors	Tocopherol: HPLC
								Fatty acids: gas chromatography
								Cytokines: ELISA
[40]	Term	Mature	Hand or electric pump	Fresh	No	62.5 °C for 30′	17 samples	Cytokines and growth factors: ELISA
[41]	Preterm and Term	Transitional and mature	N/A	Frozen	−20 °C until analysis	LABU-Muttermilch pasteurizer: 63 °C for 30′	51 samples—28 donors	IGF and IGFBP: RIA
								EGF: ELISA
[42]	Term	Mature	N/A	Fresh	No	62.5 °C for 30′	13 samples—13 donors	Free amino acids: HPLC
[43]	Preterm and Term	Colostrum, transitional and mature	N/A	Frozen	Thawed overnight	ACE pasteurizer: 62.5 °C for 30′	39 samples—3–4 donors each	Fatty acids: gas chromatography
								Free amino acids: Amino acid Analyzer
[44]	Term	Mature	Manual pump	Frozen	Refrigeration max 4 h; −20 °C up to 3 weeks	62.5 °C for 30′	5 samples—9 pools	Vitamins: HPLC
[45]	Term	Mature	Hand or pump	Fresh	Refrigeration	62.5 °C for 30′	5 each—89 donors	Vitamins: HPLC
[46]	N/A	Mature	Electric pump	Frozen	−80 °C until analysis	62.5 °C for 30′	10 samples—10 donors	Vitamin C, Tocopherols: HPLC
								Fatty acids: gas chromatography
[47]	N/A	Mature	Manual pump	Fresh	No	62.5 °C for 30′	1 pool—10 donors	Vitamin C, Tocopherols: HPLC
								Fatty acids: gas chromatography
[48]	N/A	N/A	N/A	Frozen	Frozen	63 °C for 30′	50 samples—50 donors	Vitamin A, beta catotene: HPLC
[49]	N/A	Colostrum, transitional and mature	N/A	Frozen	Thawing in ice-filled plastic container for 15′	63 °C for 30′	60 samples	Vitamin A: gas chromatography
[50]	Preterm and Term	Transitional	Electric pump	Fresh	No	62.5 °C for 30′; cooling in running water	12 samples—12 donors	Fat content: gravimetry
								Fatty acids: gas chromatography
[51]	Term	N/A	Hand	Fresh	No	62.5 °C for 30′	1 pool—16 donors	Fatty acids: gas chromatography
								L-lactate in milk: enzymatic biosensor

Table 1. *Cont.*

Ref *	Preterm/Term	Phase of Lactation	Expression Method	Status	Pre-Pasteurization Storage	Pasteurization Equipment	Sample Size	Analytical Method °
[52]	N/A	N/A	N/A	Fresh	No	63 °C for 30′	3 samples—3 donors	Total fat and fatty acids: gas chromatography, Infrared spectroscopy and NMR
[53]	N/A	Transitional	N/A	Fresh	No	62.5 °C for 30′; cooling in running water	7 samples—1 donor	Fatty acids: gas chromatography
[54]	N/A	N/A	Electric/manual pump	Fresh	N/A	62.5 °C for 30′; cooling in stirred ice-cold water bath	21 samples—21 donors	Furosine: HPLC
[55]	Preterm	N/A	Electric pump	Fresh	No	Sterifeed: 62.5 °C for 30′	10 samples—10 donors	Oligosaccharides: HPLC
[56]	Preterm	N/A	Electric pump	Fresh	No	Sterifeed: 62.5 °C for 30′	9 samples—9 donors	Glycosaminoglycans: HPLC
								Carbohydrates: gas chromatography
[57]	N/A	Mature	N/A	Fresh	Refrigeration	62.5 °C for 30′ in stirred water bath	1 pool—8 donors	Volatile compounds: gas chromatography—mass spectrometry
[58]	Term	N/A	Electric pump	Fresh	No	62.5 °C for 30′	31 samples—31 donors	MDA and GSH: HPLC
								GPx activity: Lawrence and Burk method
								ToAC: commercial kit
[59]	N/A	Mature	Hand	Frozen	−80 °C up to 2 weeks	62.5 °C for 30′	30 samples—10 donors	Total fat : creamatocrit
								Fatty acids and volatiles: gas chromatography
								MDA: TBARS
								Tocopherols and ascorbic acid: HPLC
								ToAC: ORAC
[60]	N/A	Mature	Hand	Frozen	−40 °C in HMB; −18 °C in lab	62.5 °C for 30′	1 pool—5 donors	Free nucleotide monophosphates: capillary electrophoresis—mass spectrometry

* Ref: number in reference list; ° RIA: Radioimmunoassay; ELISA: enzyme-linked immunosorbent assay; HPLC: high-performance liquid chromatography; BCA: bicinchoninic acid assay; ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); SDS-PAGE: Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis; OPA: o-phthaldialdeyde; NMR: Nuclear Magnetic Resonance; MDA: malondialdehyde; GSH: reduced glutathione; GPx: glutathione peroxidase; ToAC: total antioxidant capacity; TBARS: thiobarbituric acid reactive species; ORAC: Oxygen Radical Absorbance Capacity.

Results

A total of 58 articles were found from a combination of the searches, but only 44 fulfilled all the inclusion criteria. Table 1 summarizes the technical details on the methodological aspects of the selected articles. Table 2, Table 3 and Table 4 provide an overview of the literature on the effects of HoP on different components of DM.

Energy Content

The first question that needs to be answered concerning the nutrition of all infants with pasteurized DM is: is this milk as nutritive as fresh HM? Only two studies have been found to have assessed the effects of HoP on the energy content of milk, with conflicting results. Ley et al. [17] reported that the HM energy content was not modified by pasteurization, whereas García-Lara et al. [18] found that the energy content was decreased significantly. The two studies used different approaches to calculate the energy content; in the first case [17], a direct measurement was performed by means of bomb calorimetry, whereas in the second study the energy content was calculated on the basis of the fat, protein and lactose contents, as measured by means of infrared spectroscopy [18]. The discrepancy in the results may be due to statistical artifacts rather than to real differences, since the percent decrease measured in the two studies was similar. Overall, current evidence does not support the hypothesis of a relevant change in energy intake by feeding the infant with pasteurized rather than raw DM.

Table 2. DM components not affected by Holder Pasteurization (HoP) (consensus on results).

Components	Reference
Total Nitrogen Content	[18]
Cytokines	
IL2, IL4, IL5, IL13	[31,38]
IL12p70	[31,38,39]
IL17	[31,39]
Growth Factors	
EGF	[40,41]
TGFβ1	[40]
TGFβ2	[31]
MCP-1	[31]
Amino acids	
Free amino acids	[43]
Taurine, methionine, cystine, glutamate	[42,43]
Vitamins	
D, E, B2	[44]
B5, Biotin, B3	[45]
B12	[21,24,44,45]
Zinc	[23]
Lipids Polyunsaturated fatty acid *n*3	
20:5	[37,50]
22:5	[38,47,50]
22:6	[37,39,43,47,50]
Polyunsaturated fatty acid *n*6	
18:2	[37–39,43,47,50–53]
18:3	[39,43,47,50]
20:2	[47,50]
20:3	[39,47,50]
20:4	[37–39,43,47]
22:4	[37,47,50]
22:5	[38,47]
Monounsaturated fatty acid	
14:1	[37,39,43,47,50]
15:1	[47,50,52]
16:1	[37–39,43,47,50–53]
17:1; 22:1	[47,50]
20:1	[39,47,50]
24:1	[37,47,50]
Saturated fatty acid	
10:0, 16:0	[37–39,43,47,50–52]
15:0	[39,47,50,51]
17:0	[39,47,50]
20:0	[39,47,50,52]
21:0	[47]
22:0	[47,50]
24:0	[37,50]
Saccharides	
Oligosaccharides	[55]
Glycosaminoglycans	[56]
Myoinositol	[31,54]
Lactose	[18,19,23,31,54]
Oxidative Stress Markers	
Malondialdehyde	[58,59]
ORAC and Hexanal	[59]

Table 3. DM components affected significantly by HoP (consensus on results).

Components	Effect of HoP	References
Immunoglobulins		
IgG4	Reduction (% not reported)	[31]
Enzymes		
Lipase	Complete loss	[21,33,37]
Alkaline phosphatase	Complete loss	[21]
Amylase	Reduction (15%)	[37]
Cytokines		
IL7	Increase (% not reported)	[31]
MIP-1β	Reduction (% not reported)	[31]
MCAF/MCP-1	Reduction (% not reported)	[39]
Growth Factors		
IGF1, IGF2, IGFBP2, IGFBP3	Reduction (% not reported)	[41]
EPO	Reduction (% not reported)	[40]
HB-EGF, HGF	Reduction (% not reported)	[38]
GM-CSF	Increase (% not reported)	[31]
Hormones		
Insulin, Adiponectin	Reduction (% not reported)	[17]
Free amino acids		
Arginine, leucine	Increase	[43]
Aspartate	Reduction (% not reported)	[43]
Glutamine	Increase (% not reported)	[42]
Vitamins		
Ascorbic + Dehydroascorbic	Reduction (12%)	[47]
Ascorbic Acid	Reduction (16.2%–26%)	[46,47]
B6	Reduction (15%)	[44,46]
Oxidative Stress Markers		
Glutathione, Glutathione peroxidase activity, Total antioxidant capacity	Reduction (% not reported)	[58]
Lactulose	Increase (% not reported)/(Not detected in all samples)	[31,54]
Nucleotide monophosphate content	Increase (% not reported)	[60]

Nitrogenous Compounds

Protein Content

The composition of the HM protein fraction varies from mother to mother, and changes during lactation. The protein content of term milk is estimated to be approximately 0.9 to 1.2 g/dL, while this value is higher for preterm milk [61].

The true protein content of HM is often overestimated, due to its high proportion of non-protein nitrogen [62]. In a study by Vieirà et al. [19], the average DM protein concentration, as assessed by means of an infrared analyzer, was significantly reduced by

the HoP treatment, as was also found for colostrum [20]. Other studies, on the contrary, did not observe any significant change in protein content [17,21,22,23], even when the total nitrogen content was measured indirectly [18]. In conclusion, the majority of the examined reports indicates that HoP does not affect the protein content of DM. A statistically significant reduction was only found in two studies, although one of the studies,involving mature milk [19], reported a very slight reduction (−3.9%), similar to that found in the studies that claimed no effect on total protein content.

Table 4. DM components affected by HoP (discordant results).

Components	Effects of HoP	References
Total Protein Content	Reduction (% not reported)	Significant: [17,19,20] Not significant: [21–23,37]
Immunoglobulins		
IgA	Reduction (20%–62%)	Significant: [25,28,29,31,32] Not significant: [20,24,26,27,30,35]
sIgA	Reduction (20%–50%)	Significant: [35] Not significant: [21,34]
IgM	Reduction (50%–100%)	Significant: [28,29,31,32] Not significant: [20,24,26]
IgG	Reduction (23%–100%)	Significant: [32] Not significant: [20,24,26,28–30]

Immunoglobulins (Igs)

The effect of HoP on the DM concentration of different classes of immunoglobulins has been investigated in several studies, the first being published in 1977 by Ford and colleagues [24].

IgAs and secretory IgAs (sIgAs) are the most extensively investigated classes, and almost all the published studies report a reduction following HoP. A significant reduction in IgAs following HoP was measured by means of Enzyme-Linked Immunosorbent Assays (ELISA) [25,26,27] and by means of Radial Immunodiffusion Assays (RIA) [28,29]. An IgA reduction was also reported in older studies [24,30], although no statistical significance was reached, perhaps due to weak experimental design. Ford et al. [24] analyzed a single pooled milk sample, while Evans et al. [30] used samples derived from overflow milk. The detrimental effect of HoP on IgAs was also confirmed on colostrum samples [20,31,32]. A reduction in protein bands recognized by means of anti-IgAs antibodies, although not quantified, was also reported [33]. Secretory IgAs, the dimeric forms of IgAs, were also found to have decreased following the pasteurization of DM [21,34,35], although not always significantly.

The other Ig classes were investigated in a smaller number of studies, whose results are partially contrasting, due to the extremely low Ig concentrations, and subsequent difficult detectability in the milk samples. However, the majority of the studies found some degree of reduction.

IgM concentrations were measured in mature DM after pasteurization, and were found to be significantly decreased [26,28,29], or even completely degraded [24]. The low resistance of IgMs to pasteurization has also been confirmed more recently on colostrum [20,31,32]. The same behavior has also been observed for IgG concentrations in both milk [26,28,29,30] and in colostrum [20,31,32]. The specific thermal resistance of different IgGs subclasses was also detailed: IgG1 were not affected by HoP, while IgG4 were reduced, and IgG2 and IgG3 were undetectable in both fresh and pasteurized milk samples [31].

As an overall conclusion, the results from the previous reports clearly indicate that one of the main detrimental effects of HoP is a reduction in all classes of immunoglobulins, probably due to the complex structure of these molecules.

Lactoferrin and Lysozyme

The immunoprotective protein constituents of HM, with bacteriostatic properties, include lysozyme and lactoferrin [62].

Lactoferrin is an iron-binding protein that reduces the availability of the free iron required by iron-dependent pathogens, and therefore is able to inhibit their growth. Moreover, it can disrupt the bacterial cell membrane by binding to the lipid-A portion of lipopolysaccharides on the bacterial cell surface [63]. Lactoferrin has been investigated in several studies, using different techniques (ELISA, RIA, monospecific antisera) [24,29,30,31,32], all of which report a reduction in its concentration, with a percentage ranging from 35% to 90%, but the reduction was only reported as significant by Christen et al. [35]. Additionally, a reduction in the lactoferrin-containing band, by means of a protein electrophoresis semi-quantitative method, was reported [33]. Since the bactericidal activity of lactoferrin is maintained by bactericidal peptides that form during its digestion, it is possible that part of the activity is still retained in pasteurized HM despite reduction of the protein [62]. One recent survey [64] conducted by means of non-reducing protein electrophoresis, has reported that lactoferrin aggregation, rather than degradation, occurs following DM pasteurization with HoP. Whether this aggregation causes a decrease in lactoferrin bactericidal activity, is still not known.

A similar pattern emerges for lysozyme, whose concentration has been found to be reduced after pasteurization in several studies, with a percentage ranging from 20% to 85%. The biological activity of lysozyme was tested by Ford et al. [24], who did not find any significant difference. A significant reduction in lysozyme activity after HoP was found by other authors [25,36], even in colostrum [32]. It should be pointed out that, in those works, the lysozyme activity was always determined using a Micrococcus lysodeikticus-based turbidimetric assay, which measures to what extent bacterial growth is prevented by the addition of lysozyme-containing samples. Since DM is a complex mixture of several anti-bacterial enzymes and factors, it is not possible to discriminate if the reduction is due only to a decrease in lysozyme concentration.

Table 4. *Cont.*

Components	Effects of HoP	References
Lactoferrin	Reduction (35%–90%)	Significant: [35] Not significant: [24,29,30,33,34]
Lysozyme		
concentration	Reduction (20%–69%)	Significant: [34] (Sterifeed and Carag), [35] Not significant: [20,21,30,34] (Saurin)
activity	Reduction (% not reported)	Significant: [25,32] Not significant: [24]
Cytokines		
IL1beta, IL6	Reduction (% not reported)	Significant: [39] Not significant: [31]
IL8	Increase (% not reported)	Significant: [38–40] Not significant [31]
IL10	Reduction (% not reported)	Significant: [38,39] Not significant: [31]
TNF alfa	Reduction (% not reported)	Significant: [38,39] Not significant: [31]
INF gamma	Reduction (% not reported)	Significant: [38] Not significant: [31,39]
Vitamins		
A	Increase (% not reported)/reduction (34%)	Significant: [49] Not significant: [23,44,48]
Folacin	Reduction (10%–30%)	Significant: [44] Not significant: [21,24,29]
C	Reduction (19.9%–36%)	Significant: [44,46] Not significant: [29]
alfa- and gamma-Tocopherol	Reduction (12%–47%)	Significant: [39,47] Not significant: [46]
delta-Tocopherol	Reduction (% not reported)	Significant: [39] Not significant: [46]
Total fat content	Reduction (% not reported)/Increase (% not reported)	Significant: [17–19] Not significant: [23,37,50,51]
Polyunsaturated fatty acid *n*3		
18:3	Reduction (% not reported)	Significant: [53] Not significant: [38,39,47,50]
Monounsaturated fatty acid		
18:1	Increase/reduction (% not reported)	Significant: [38] Not significant: [39,47,50,53]
Saturated fatty acid		
14:0	Increase/reduction (% not reported)	Significant: [38] Not significant: [37,39,43,47,50,52,53]
12:0	Increase/reduction (% not reported)	Significant: [38,43] Not significant: [37,39,47,50–52]
18:0	Increase/reduction (% not reported)	Significant: [38] Not significant: (2015) [37,39,43,47,50–52]
Glucose	Reduction/Increase (% not reported)	Significant: [17] (increase), [54] (reduction) Not significant: [31]

Other Enzymes

Owing to their different responses to heating, enzymes and their activity are commonly considered as markers for assessment of thermal treatments. Some of these enzymes (such as lactoperoxidase and alkaline phosphatase) are considered technological mark-

ers for pasteurization in bovine milk. Data on lactoperoxidase are scarce for HM, since its concentration is below the detection limit of commercial kits [24,32]. Alkaline phosphatase has also been found to be completely inactivated by HoP [21], as commonly found in bovine milk.

Because of the compensatory function of several HM enzymes for nutrient digestion in newborns [65], some researchers have focused on the activity of lipase and amylase milk enzymes. A complete degradation (both in concentration and enzymatic activity) has been found for lipoprotein lipase and bile salt dependent lipase [21,33,37], while amylase activity was partly retained [37]. The clinical relevance of variations in the enzymatic activity of these proteins still has to be investigated. In particular, the hypothesis of a reduction in nutrient absorption through feeding of pasteurized rather than raw HM, especially as far as lipid digestion is concerned, cannot be ruled out.

Cytokines

Although cytokines are immunomodulatory components, it appears that most of those found in HM are anti-inflammatory, thereby possibly lessening the effect of infections [62]. The effect of pasteurization on several cytokines has been evaluated in colostrum [31]. Interleukin (IL)1β, IL2, IL4, IL5, IL6, IL8, IL10, IL12, IL13, IL17, Interferon (IFN)-γ, the Tumor Necrosis Factor (TNF)-α and Monocyte Chemotactic Protein (MCP)-1 were not significantly affected by the process. Rather remarkably, IL7 increased significantly after pasteurization, perhaps due to its release from cellular and/or fat compartments into the aqueous fraction, while Macrophage Inflammatory Protein-1β (MIP-1β) was significantly reduced [31]. IL2, IL4, IL5, IL12 and IL13 were unaffected even in pasteurized mature DM [38], while IL10 was decreased [38,39,40], as were IL1β [38], IFN-γ [39], IL6 [39] and TNF-α [38,39]. A significant increase in IL8, following HoP in mature DM, was also found [38,39].

In short, different degrees of thermal resistance were found for different cytokines, and the biological relevance of this altered balance in specific situations still remains to be addressed.

Growth Factors

To date, only a few studies have evaluated the variation of growth factors (GF) in human milk after pasteurization, and each one has focused on one specific GF. Transforming GF (TGF)-β2 was found to be stable in colostrum [31]. Epidermal GF (EGF) [40,41] and TGF-β1 [40] showed no difference before and after HoP. Heparin-Binding Epidermal-like GF (HB-EGF) was unaffected by heating, whereas Hepatocyte GF (HGF) was reduced to a great extent [38]. Insulin-like GF (IGF)-1 and 2, as well as IGF binding proteins 2 and 3, were reduced to a variable extent by pasteurization [41]. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) concentrations increased significantly after pasteurization, while the Granulocyte-Colony Stimulating Factor (GCSF) was not

detected in any sample, before or after HoP, either in colostrum [31], or in mature milk [38].

Once again, as in the case of cytokines, the low number of studies to date, and the relevant variability between different growth factors, does not allow any conclusion on the effect of HoP to be generalized.

Amino Acids

The DM amino acid composition was investigated in two studies, one [42] focusing only on sulfur amino acids (cysteine and methionine), and a few free amino acids. The authors reported that no significant modification was caused by HoP, with the exception of an increase in free glutamine [42]. In another study [43], the amino acid profile of pre- and post-pasteurization DM was found to be significantly different for arginine and leucine, to increase following HoP, and to decrease for aspartate. Nevertheless, the biological relevance of these variations was reported to be low, since the differences, although significant, were small.

The available lysine content, which is considered a nutritional marker, as lysine residues are targeted by proteases during digestion, was evaluated in two studies, with discordant results: Silvestre et al. [22] showed a 30% reduction (statistically significant) using a fluorimetric method, while Baro et al. [33] observed an increase in the concentration of this amino acid, measured by means of o-phthaldialdehyde. In this case, the discordant results may partially be due to the different experimental designs of the two studies, one of which simulated HoP [22], and the other used a commercial Holder pasteurizer [33].

Hormones

Insulin, adiponectin [17] and erythropoietin [40] concentrations were all reported to be decreased significantly after HoP, although the paucity of the reports does not allow any conclusion on the issue to be generalized.

Vitamins

The evidence on the effect of HoP on HM vitamins is contrasting. Folacin and vitamin B12 were evaluated in different studies with conflicting results, probably due to the significant instability of vitamins and to the variability between the study sampling and analysis methods of the studies. Ford et al. [24] found an important reduction in the capacity of DM to bind added vitamins after HoP, as measured by adding excess cyanocobalamin (vitamin B12 analog), while other studies [21,44] did not find any significant difference for vitamin B12 analyzed by means of a chemiluminescence immunoassay and a competitive protein binding assay. Stability of riboflavin, biotin, and the total pantothenic acid contents (vitamin B5) has also been observed [45]. A non-significant decrease was found for folacin (vitamin B9) after HoP [21,24,45]. Van Zoeren-Grobben

et al. on the other hand, found a 30% reduction in folacin as a consequence of HoP, and a significant 15% reduction in vitamin B6 [44].

While the vitamin C concentration was unambiguously reported to be reduced by HoP [44,46,47], fat-soluble vitamins showed different responses to pasteurization. Vitamin D was unaffected [44]. In some reports, vitamin A was found to be reduced [48,49], but other authors found it stable [23,44], while tocopherols (vitamin E) were found not to be affected in [44,46], but reduced in [39,47].To summarize, the available data seem to confirm a higher heat sensitivity for water soluble vitamins, and in particular for vitamin C, which is known to be highly susceptible to several technological treatments, including freezing and refrigeration [66], while most of the studies seem to indicate a higher retention of fat soluble vitamins (A, D, and partially E).

Zinc

Zinc levels have not resulted not to be significantly affected by HoP; however, a variation in the distribution of zinc was observed, with a significant increase in the fat fraction and a decrease in whey, possibly as a result of the denaturation of zinc binding proteins, thereby indicating possible consequences on zinc bioavailability to the infant [23].

Lipids

The total lipid content was evaluated in several studies: a significant reduction was found following HoP, using infrared analyzers [17,18,19], which, however, do not directly measure the fat content. Other authors have not found significant differences, when using different analysis techniques [23,37,50,51]. The total fatty acid profile was always found to be unaffected by pasteurization [37,39,43,47,50,52], with the exception of one study, which reported slight changes in the relative composition of medium-chain fatty acids (MCFAs) [38], and one of which reported a small decrease in 18:3 fatty acid [53]. Lepri et al. [51] found a more than two-fold free fatty acid content increase after pasteurization. A potential increase in the DM free fatty acid fraction following HoP, provided it does not cause off-flavors, may not be undesirable, as increased free fatty acids are known to be more readily absorbed in the digestive system, thus resulting in a possibly increased nutritive potential.

Saccharides

Several studies have evaluated the effect of HoP on lactose in mature milk and colostrum. In all cases, and using different analytical techniques, no significant difference was found before and after HoP [18,19,23,31,54]. The myoinositol levels were not affected by HoP either [31,54], while glucose was found to be increased [17], stable [31] and reduced [54], although all of the reported variations were low.

Glycosaminoglycans and oligosaccharides have recently been investigated in preterm milk, and no variation after the pasteurization process was observed [55,56]. These human milk glycans have been demonstrated to influence the health of newborns, since they possess specific biological properties, such as an anti-infective role, anti-oxidant functions and prebiotic effects [67].

In short, the evidence to date points toward the stability of DM saccharides during pasteurization by HoP, both as free molecules and as part of biologically active compounds, such as glycosaminoglycans and oligosaccharides.

Indicators of Thermal Treatment

Lactulose, a disaccharide formed through the isomerization of lactose, and furosine, an intermediate of the Maillard reaction, have been searched for in DM, since they are used in the food industry to differentiate between different kinds of heat-treated bovine milk, depending on the intensity of the heat treatment applied. Since HM has more than double the lactose content than bovine milk, lactulose formation has been found to be triggered more by pasteurization than is normally found for pasteurized bovine milk, in both milk [67] and colostrum [31]. Furosine was only found to have formed following pasteurization in colostrum samples [31], while it was not found in any milk sample [67]. Contador et al. [57] have very recently published a detailed analysis of volatile compounds in fresh and pasteurized HM, which supports the idea of some thermal-induced modification during HoP. The preservation of the original volatile compounds in HM is important, since they can negatively affect its quality, and indicate that undesired reactions have taken place during the treatment (e.g., lipid oxidation, Maillard reaction, etc.). A significant change in many volatiles was detected after HoP in DM; some of these volatiles (aldehydes, furans and pyrans) are undesired, and considered an index of thermal degradation.

Oxidative Stress Markers

The oxidative balance of raw and pasteurized HM was assessed by measuring both the accumulation of oxidants and the activity of oxidant scavengers. Malondialdehyde concentrations were not found to be significantly affected by HoP but, on the other hand, glutathione concentrations, glutathione peroxidase activity and Total Antioxidant Capacity were significantly reduced, thus indicating a decrease in the oxidative scavenging capacity of HM [58]. In a more recent assay [59], no change in malondialdehyde concentration, total antioxidant capacity (as measured by means of Oxygen Radical Absorbance Capacity assay), or hexanal concentration has been found, thus indicating no lipid oxidation and no decrease in oxidant scavengers.

Organic Acids

Only one study has assessed the l-lactate content of HM, following HoP, using a biosensor based on an immobilized lactate oxidase enzyme. The results showed a significant increase, probably due to the release from interaction with other substances [51].

Recently Published Research

One study has recently highlighted an increase in the nucleotide monophosphate content following pasteurization, measured by means of mass spectrometry. These compounds are considered as immune-enhancers and seem to play a role as sleep-inducers, so their increase in HM may be a positive consequence of HoP [60]. Nevertheless, further evidence is needed before any claim on the issue could be formulated.

Discussion and Conclusions

The present review shows a significant variability in the data reported in the scientific literature concerning the effects of HoP on the biological components of HM. A possible explanation for this variability may be the heterogeneity of the test protocols applied in the studies (e.g., in terms of sample origin, storage conditions or analysis methods). Another important source of variability is represented by the fact that the Holder pasteurization of donor milk is often simulated on small aliquots, rather than being performed following HMB-implemented protocols. Moreover, modern pasteurizers require significantly less time for heating and cooling than older ones, thus changing the kinetics of the thermal response for heat-sensible compounds. Additionally, it appears that some biochemical patterns were investigated more extensively than others, while some other milk components were not considered at all.

The results of the review can be summarized as follows. Saccharides are not significantly affected by the heat treatment, as either free molecules or as part of biologically active compounds. The total lipid content is preserved by HoP, as is its fatty acid composition. This finding is of paramount importance since it suggests that pasteurization is able to preserve both the nutritional and biological properties relevant to the development of the central nervous system associated with some of these fatty acids. Consistently, fat soluble vitamins also seem to be unaffected, while water soluble vitamins, and vitamin C in particular, are generally reported as significantly decreased. The results concerning specific biologically active molecules (such as cytokines and growth factors) remain uncertain, due to the vast number of different compounds analyzed in each study, and to the paucity of comparable results.

Proteins are more significantly affected by HoP. In fact, specific proteins with significant immunologic and anti-infective action (such as immunoglobulins and lactoferrin) are reduced by pasteurization. A substantial reduction in the enzymatic activity has also

been observed. The review thus confirms the main concerns about Holder pasteurization of HM, and the need for future strategies to prevent and/or limit DM protein degradation. The available data confirm that HoP affects several HM components to variable degrees, even though it is rather difficult to quantify the degradation level. Nonetheless, clinical practices demonstrate that many beneficial properties of human milk remain, even after pasteurization.

Future studies should be aimed at confirming the currently available data by investigating more reproducible analytic settings, while avoiding the introduction of potential biases, in order to understand the real effects of pasteurization on mother's milk. Moreover, further studies should be focused on new pasteurization techniques in order to improve the biological quality and safety of DM.

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Chapter 2

Effects of Holder pasteurization on the protein profile of human milk

CHIARA PEILA[1], ALESSANDRA COSCIA[1], ENRICO BERTINO[1], MARIA CAVALETTO[2], STEFANO SPERTINO[2], SARA ICARDI[2], CLAUDIA TORTONE[1], GERARD HA VISSER[3] AND DIEGO GAZZOLO[4]

1Neonatology Unit, Department of Public Health and Pediatrics, University of Turin, Italy; 2DiSIT, Dept. of Science and Technological Innovation, University of East Piedmont "A. Avogadro"; Alessandria, Italy; 3Department of Obstetrics, University Medical Center, Utrecht, the Netherlands; 4Department of Maternal, Fetal and Neonatal Health, C. Arrigo Children's Hospital, Alessandria, Italy.

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Abstract

Background: The most widespread method for the treatment of donor milk is the Holder pasteurization (HoP). The available literature data show that HoP may cause degradation of some bioactive components. The aim of this study was to determine the effect of HoP on the protein profile of human milk (HM) using a GeLC-MS method, a proteomic approach and a promising technique able to offer a qualitative HM protein profile.

Methods: HM samples were collected by standardized methods from 20 mothers carrying both preterm and term newborns. A aliquot of each sample was immediately frozen at -80°C, whilst another one was Holder pasteurized and then frozen. All samples were then analyzed by GeLC-MS. The protein bands of interest were excised from the gel, digested with trypsin and identified by nano-HPLC-MS/MS analysis.

Results: The protein profile before and after HoP showed qualitative differences only in 6 samples out of 20, while in the remaining 14 no detectable differences were found. The differences interested only colostrums and transitional milk samples and regarded the decrease of the electrophoretic bands corresponding to alpha and beta-casein, tenascin, lactoferrin and immunoglobulin.

Conclusions: In the majority of samples, HoP did not cause any modification, thereby preserving the biological activity of HM proteins.

Key Words: Human milk; Donor Human Milk; Pasteurization Holder; GeLC-MS; Tenascin.

Introduction

Human milk (HM) is considered the "gold standard" nutrition for feeding term and preterm newborns [1]. When mother's own milk is not available or not sufficient, despite significant lactation support, Donor Human Milk (DM) is an important and the first alternative, especially in high risk newborns admitted to Neonatal Intensive Care Units (NICU) [2,3]. The main benefit deriving from the use of DM versus formula milks, in preterm infant feeding, is the reduction in the incidence of necrotizing enterocolitis (NEC) as shown by several meta-analysis [4-7]. Moreover, an enhanced feeding tolerance has also been reported [3,8].

The DM must be collected, processed and stored ensuring its microbiological safety maintaining at the same time the nutritional quality (NQ) [9]. NQ control of HM storage is complex as well as mandatory and pasteurization procedure is the main processing step to inactivate pathogenic microorganisms [2,10]. DM is typically pasteurized by Holder Pasteurization method (HoP) characterized by heating at 62.5 °C for 30 minutes. HoP is currently the requested procedure by the majority of Human Milk Banking Associations (HMBA), being a temperature below 62,5°C considered not safe [2,10]. The impact of HoP on the biological quality of HM has been investigated and results are still controversial and matter of debate. The main explanations reside in the different procedures regarding samples collection, storage and treatment [11-13]. In this regard, data on the total protein count and specific proteins have been provided by using immune-enzymatic techniques such as ELISA. Recently, GeLC-MS, a proteomic approach involving the separation of proteins in SDS-PAGE by one-dimensional electrophoresis (1DE) followed by identification by mass spectrometry (MS), has been suggested as a promising technique able to offer a qualitative HM protein profile [14]. Data on GeLC-MS pattern in HM previously treated by HoP and later on stored at Human Milk Bank (HMB) are still lacking.

Therefore, the purpose of the present study was to determine the effect of HoP on the protein profile of HM by using GeLC-MS analysis, under reducing and non-reducing conditions, in order to investigate the qualitative HM protein profile and the occurrence of protein aggregates, eventually formed after heat treatment.

Methods

Sample collection

We conducted a pretest–test design, where HM samples acted as their own controls. Breast milk was collected, at different stage of maturation (colostrum: n=9; transitional milk: n=5; mature milk: n=6) according to Playford et al. [15], from 20 mothers delivered between 23 and 41 weeks of gestational age (GA). The study was approved by local

ethic committee and mother gave informed and signed consent to the study. Exclusion criteria were: maternal infections, tobacco smokers, drugs addiction and alcoholic; use of drugs or pharmacologically active substances; mothers who received blood transfusions or blood products, or organ transplants; fetal malformations, chromosomal abnormalities, perinatal asphyxia and dystocia. HM samples were collected at two consecutive mornings, between 8-9 a.m., into disposable high density polyethylene sealed bottles (Flormed, Napoli, Italy) sterilized by using ethylene oxide. Milk expression was obtained by emptying one or two breasts with an electric breast pump (Medela Symphony). From each container, 10 mL of HM were taken, divided into two fractions: the first was immediately frozen at -80°C; the second was pasteurized in HMB and frozen at -80°C. HoP was performed with a Sterifeed Pasteuriser by Medicare Colgate Ltd (Cullompton, England), heating milk at 62.5°C for 30 minutes. The last HoP phase requires a rapid and precise cooling of milk samples to 10°C in approximately 20 minutes, by immersion into cold water.

Sample preparation and protein quantification

Skimmed HM samples were obtained by centrifugation at 2000 x g for 30 min at 10°C, the pellet and the floating layer were discarded. Protein content was estimated according to Bradford [16].

GeLC-MS analysis and protein identification

Skimmed milk samples were mixed with Laemmli buffer (2% w/v SDS, 10% Glycerol, 5% β-mercaptoethanol, 62 mM Tris-HCl pH 6.8), boiled for 5 min, and loaded on 10 x 8 cm vertical 12% polyacrylamide gels. For non-reducing conditions the Laemmli buffer did not contain β-mercaptoethanol and samples were not boiled. SDS-PAGE was performed at 10 mA per gel for 30 min and 30 mA per gel until the tracking dye front reached the bottom of the gel, at 10°C with a Mini Protean II Xi System (Bio-Rad). The running buffer was 25 mM Tris-HCl, 200 mM Glycine, 0.1% w/v SDS. The gels were stained overnight with Colloidal Coomassie brilliant blue G250 (Bio-Rad Laboratories) in accordance with Neuhoff et al. [17]. The Coomassie-stained gels were scanned using an Image Scanner III (GE Healthcare) at 300 dpi. The protein bands of interest were manually excised from 1DE gels and in-gel digested with trypsin as described by Spertino et al. [18]. The peptide mixtures were pooled and lyophilized in a SpeedVac for mass spectrometry analysis.

MS/MS analysis was performed using a QSTAR XL hybrid quadrupole–TOF instrument (Applied Biosystems, Foster City, CA, USA) coupled with a LC Packings Ultimate 3000 nano-flow LC system (Dionex, Amsterdam, The Netherlands), as described by Bona et al. [19]. Briefly, the QSTAR XL operated in positive mode and in information-dependent acquisition (IDA) mode, the dynamic exclusion feature of the Analyst QS 1.1 software (Applied Biosystems, Foster City, CA, USA) was enabled, with an exclusion

mass width of +/- 3 m/z for 60s. LC/MS–MS files obtained from each protein sample were merged into a single MASCOT generic format (mgf) file and searched against the NCBI non-redundant database; tolerance for precursor and fragment masses was 0.25 Da. The proteins were identified in homology with significant ion scores ($p<0.05$).

Statistical Analysis

Clinical data are reported as the mean and SD. Protein content (mg/mL) are reported as median and interquartile ranges. Statistical analysis was performed using XLStat-Pro v.7.2.5 (Addinsoft, New York, USA). Results were compared between groups by Mann–Whitney U-two sided test when the data did not follow a Gaussian distribution. A value of $P<0.05$ was considered significant.

Results

Demographic characteristics of milk donors

The demographic characteristics of the milk donors are shown in Table 1. As expected, the incidence of delivery mode and the need of caesarean section were within the reference for our country. Gestational age and maternal age at birth were within reference curve for our national standards. All mothers showed normal clinical conditions. No overt neurological injury and/or infections were observed at the sampling time-points or at discharge from the hospital.

Table 1. Demographic characteristics of milk donors.

Donor	Mother's ethnic group	Age (yrs)	Previous breastfeeding	GA (wks)	Delivery Mode	Milk maturity degree	Spontaneous Pregnancy
1	Caucasian	38	Yes	28	CS	Mature	Yes
2	Caucasian	31	Yes	25	V	Transitional	Yes
3	Caucasian	38	Yes	35	C	Transitional	Yes
4	Caucasian	35	Yes	31	CS	Mature	Yes
5	Caucasian	35	No	23	CS	Mature	No
6	Caucasian	39	No	35	V	Transitional	Yes
7	Caucasian	37	No	27	V	Transitional	Yes
8	Caucasian	25	No	29	CS	Mature	Yes
9	Caucasian	31	Yes	32	CS	Mature	Yes
10	Caucasian	33	No	33	CS	Transitional	Yes
11	Caucasian	30	No	31	CS	Mature	No
12	Caucasian	22	No	38	V	Colostrum	Yes
13	Other	33	Yes	40	V	Colostrum	Yes
14	Caucasian	28	No	38	V	Colostrum	Yes
15	Caucasian	34	No	39	CS	Colostrum	Yes
16	Caucasian	40	Yes	38	V	Colostrum	Yes
17	Caucasian	39	Yes	37	V	Colostrum	Yes
18	Caucasian	36	No	37	V	Colostrum	Yes
19	Caucasian	37	Yes	37	V	Colostrum	Yes
20	Caucasian	33	No	41	V	Colostrum	Yes

Abbreviations. Years (yrs); weeks (wks); caesarean section (CS); vaginal (V).

Total protein content determination

The data of protein content are reported in No-Holder pasteurized (NO-HoP) and Holder pasteurized (HoP) groups in Table 2 and in Table 3. Both in NO-HoP than in HoP groups no significant differences (P>0.05, for all) have been found in total protein content, also after correction for milk maturity degree (Table 2).

Table 2. The median of protein content in human milk samples before (No-HoP) and after Holder pasteurization (HoP). Data are given in median and interquartile ranges.

Parameters	No-HoP			HoP		
	Median	25%	75%	Median	25%	75%
All milk samples (n=20)	14.49	10.28	17.22	11.96	10.21	15.65
Colostrum (n=9)	14.58	13.69	20.32	12.22	11.32	17.72
Transitional (n=5)	14.89	10.42	16.76	14.50	10.63	15.52
Mature (n=6)	11.30	8.91	16.71	9.96	8.35	12.00

Table 3. Protein content (mg/ml) in human milk samples before (No-HoP) and after Holder pasteurization (HoP).

Sample	No- HoP	HoP
1	12,99	10,16
2	9,99	10,76
3	17,85	14,50
4	8,92	12,00
5	8,02	7,62
6	14,89	15,39
7	10,57	10,27
8	16,71	8,36
9	29,10	30,13
10	16,40	15,92
11	9,63	9,77
12	29,42	21,87
13	13,09	7,82
14	14,59	11,11
15	13,89	12,23
16	14,41	11,92
17	16,29	15,14
18	28,05	31,86
19	9,83	11,40
20	17,74	16,34

Proteomic analysis and protein identification

The separation of proteins using 1DE permits to visualize the various protein species in a biological sample; Figure 1 shows electrophoretic separation of milk's proteins, under reducing and non-reducing conditions.

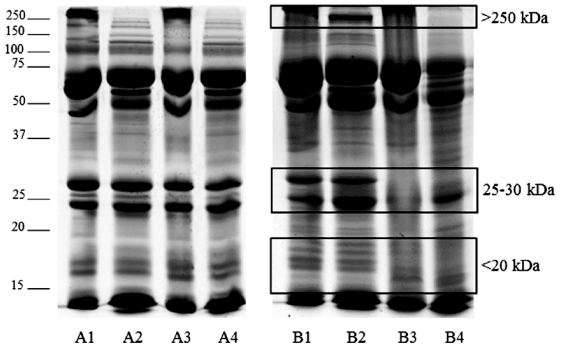

Fig. 1 Representative 1DE profiles of pasteurized milks. Legend. Sample A: milk unchanged after Holder pasteurization; sample B: milk changed after Holder pasteurization. For each sample, lane 1: raw milk under non-reducing conditions; lane 2: raw milk under reducing conditions; lane 3: pasteurized milk under non-reducing conditions; lane 4: pasteurized milk under reducing conditions. Differences in band profiles were observed in three molecular weight regions, indicated by the squares

Six out of 20 samples, corresponding to colostrum (n=5) and transitional milk (n=1), showed a reduction of band intensity whilst no changes were observed in the mature milk samples. There were no differences in the six samples after correction for delivery mode and/or gestational age at birth. Reduction of intensity was observed in three regions: <20kDA; 25-30kDa; >250kDa. Concerning the bands in the region between 25 and 30 kDa, the distribution of aggregates between protein fragments was altered by pasteurization in five milk samples, individually or in association with other modifications.

The proteins identified by MS in the different regions (Figure 2), are shown in Table 4.

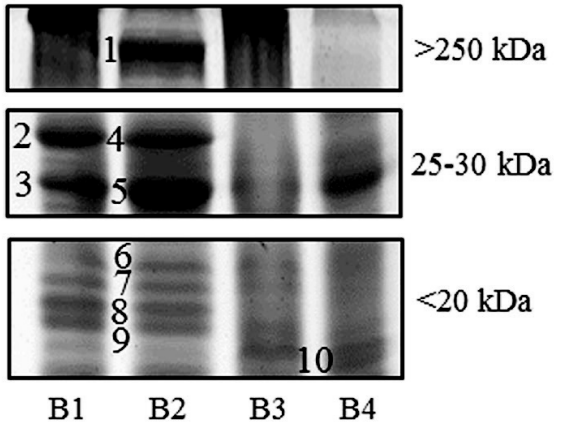

Fig. 2 1DE detail of changed protein bands in the different molecular weight regions. Legend. Numbers indicate the protein bands identified by MS/MS analysis

Table 4: List of proteins identified from bands showing changes after Holder pasteurization.

Band no.	Protein ID	AC number (gi NCBI)	Theoretical Mr (kDa)	MASCOT score	Sequence coverage (%)	MS/MS sequencing
1	TNC variant protein	68533131	244.2	1177	15%	R.LEELENLVSSLR.E
						K.FTTDLDSPR.D
						R.ELEPGVEYFIR.V
						R.VFAILENKK.S
						R.VATYLPAPEGLK.F
						R.QTGLAPGQEYEISLHIVK.N
						R.LDAPSQIEVK.D
						K.ETFTTGLDAPR.N
						R.VSQTDNSITLEWR.N
						K.TTLTGLRPGTEYGIGVSAVK.E
						K.EDKESNPATINAATELDTPK.D
						K.ESNPATINAATELDTPK.D
						R.GLEPGQEYNVLLTAEK.G
						K.AATPYTVSIYGVIQGYR.T
						R.AVDIPGLEAATPYR.V
						R.TPVLSAEASTAK.E
						K.QSEPLEITLLAPER.T
						R.EATEYEIELYGISK.G
						R.APTAQVESFR.I
						K.FTTDLDSPR.D
						R.DLTATEVQSETALLTWRPPR.A
						K.EVIVGPDTTSYSLADLSPSTHYTAK.I
						K.IQALNGPLR.S
						R.EEFWLGLDNLNK.I
						K.ITAQGQYELR.V
						R.DHGETAFAVYDKFSVGDAK.T
2	Beta-casein	288098	25.2	63	12%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
3	Beta-casein	288098	25.2	65	12%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
4	Lactotransferrin	16198359	78.3	333	8%	R.YYGYTGAFR.C
						R.THYYAVAVVK.K
						R.SDTSLTWNSVK.G
						R.CLAENAGDVAFVK.D
						R.RSDTSLTWNSVK.G
						K.LRPVAAEVYGTER.Q
	Immunoglobulin kappa light chain VLJ region	21669409	29.8	118	17%	R.TVAAPSVFIFPPSDEQLK.S
						K.DSTYSLSSTLTLSK.A
						K.VYACEVTHQGLSSPVTK.S
	Beta-casein	288098	25.2	106	28%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
						R.AVPVQALLLNQELLLNPTHQIYPVT
						QPLAPVHNPISV.-

Table 4: Continued

Band no.	Protein ID	AC number (gi NCBI)	Theoretical Mr (kDa)	MASCOT score	Sequence coverage (%)	MS/MS sequencing
4	Lactotransferrin	16198359	78.3	333	8%	R.YYGYTGAFR.C
	Immunoglobulin lambda light chain	33700	25.2	77	20%	R.SYSCQVTHEGSTVEK.T
						K.YAASSYLSLTPEQWK.S
						K.AAPSVTLFPPSSEELQANK.A
5	Immunoglobulin kappa light chain VLJ region	21669357	29.5	381	34%	K.LLIYWASTR.E
						K.DSTYSLSSTLTLSK.A
						K.SGTASVVCLLNNFYPR.E
						K.LYACEVTHQGLSSPVTK.S
						R.TVAAPSVFIFPPSDEQLK.S
						K.VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK.A
	Alpha S1-casein	1359714	20.7	192	19%	R.LQNPSESSEPIPLESR.E
						R.LNEYNQLQLQAAHAQEQIR.R
	Immunoglobulin lambda light chain	33700	25.2	139	14%	K.YAASSYLSLTPEQWK.S
						K.AAPSVTLFPPSSEELQANK.A
	Beta-casein precursor	4503087	25.4	115	11%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
6	Beta-casein	288098	25.2	49	12%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
7	Alpha S1-casein	1359714	20.7	56	10%	R.LNEYNQLQLQAAHAQEQIR.R
	Beta-casein	288098	25.2	50	12%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
8	Beta-casein	288098	25.2	38	12%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
9	Beta-casein	288098	25.2	81	12%	K.SPTIPFFDPQIPK.L
						K.VLPIPQQVVPYPQR.A
	tenascin	37227	240.6	70	1%	R.LEELENLVSSLR.E
	alpha S1-casein	1359714	20.7	65	10%	R.LNEYNQLQLQAAHAQEQIR.R
10	Lactoferrin	186833	78.3	203	3%	R.THYYAVAVVK.K
						K.LADFALLCLDGK.R
						K.NLLFNDNTECLAR.L
	Beta-casein	288098	25.2	38	6%	K.VLPIPQQVVPYPQR.A

Discussion

When mother's own milk is not sufficient, donor milk is the first best choice for feeding term and preterm newborns, due to its well-recognized nutritional advantages with respect to formula milk [4-8]. DM should be obtained from established HMBs that follow specific guidelines for screening, storage, and handling procedures to optimize its composition while ensuring its safety for the recipient. [2,10] All the milk arriving to the Human Milk Bank must be pasteurized. The ideal pasteurization process should consist of a phase of rapid heating followed by a phase of constant maintenance of the temperature and a final phase of rapid cooling. Pasteurization of the milk minimizes the risk of disease transmission via HM, inactivating most of the viral and bacterial contaminants. In addition, donors are screened in a similar way as for blood donation. No report has been published showing transfer of diseases through pasteurized DM, although milk may contain microorganisms. [2,10]. Currently Holder Pasteurization (62.5°C for 30 minutes) is the gold standard for milk processing in Human Milk Banks and is recommended by several guidelines as the optimal compromise between quality and microbiological safety [2,10]. Several studies have already been performed to evaluate the effects of pasteurization on mother's milk macronutrients; the related results are often discordant, especially concerning proteins [8,20-22].

Our study observed a non-significant reduction of the total protein count following HoP of milk samples. This result is in line with the available literature: several studies reached similar conclusions using different analytical methods [20,21]. Only Vieira et al. in 2011 found that there was a significant reduction in the mean protein concentrations, between the raw and post-pasteurization samples (reduction of 3.9%). However, these samples were only analyzed with the use of a FT-IR infrared analyzer [22].

The original contribution of our study consists of the use of a semi-quantitative analytical method, GeLC-MS analysis. This technique allows to evaluate the protein profile of human milk, which is constituted by a complex array of biologically active proteins. Each sample was tested and compared under reducing and nonreducing conditions, to highlight the possible presence of protein-complexes due to disulfide bond formation in the pasteurization step. However this method is not able to evaluate the protein changes associated whit interaction between proteins and sugars, or proteins and lipid due to thermic treatments. No differences were observed between the electrophoretic profile of the same sample under reducing and nonreducing conditions, except for the region of high molecular weight, probably corresponding to the formation of high-mass complexes which do not run in the gel. Over 75 kDa, we observed more numerous and well separated bands under reducing conditions. The peculiarity in our data consists of the observation that no variation is present in 14 out of 20 samples (previous studies have detected a variation in all samples). It is also noteworthy that amongst the 6 samples presenting a modification in the protein levels, 5 derived from colostrum milk and only 1 from transitional milk. This finding is relevant from a clinical practice standpoint, since

donor milk usually consists of mature milk (which, according to our data, did not show any variation). Each band showing a variation was then identified with the corresponding protein(s). The bands of greatest interest are the ones of medium molecular weight (25-30 kDa) since they contain beta casein, alpha-casein, lactoferrin fragments and immunoglobulin light chains (Igκ and Igλ); the other band of interest (250 kDa) was identified as tenascin.

Tenascin is a homohexameric disulfide-linked glycoprotein and its expression decreases with lactation. This protein was investigated in recent studies for its antimicrobial properties in milk and Fouda et al. have hypothesized its ability to neutralize the HIV-1 virus via binding to the chemokine receptor site [25]. Tenascin has never been evaluated previously in human milk after pasteurization and we observed a reduction only in reduced samples of colostrum.

Beta-casein and alpha-casein are two important proteins of human milk. Only one previous study has investigated the effect of Holder pasteurization on these proteins using an electrophoretic analysis method and has shown a slight decrease in a single pooled sample [14].

IgA antibodies in breast milk provide passive immunity and sufficient protection against infections to neonates and preterm infants [24]. Lactoferrin is a bioactive protein that serves as an immunomodulatory factor [24]. Several studies revealed a number of functions of these molecules: i) IgA has specific antigen-targeted anti-infective action; ii) lactoferrin plays important roles in immunomodulation, iron chelation and antimicrobial action, and exhibits the anti-adhesive and trophic properties necessary for intestinal growth [24-25]. Overall, regarding lactoferrin and IgA, our study is partially in agreement with the results reported in the literature, in which a decrease was found in all samples tested, in contrast to our findings. There might be two reasons for these differences: i) we did not use pooled milk (as done in several previous studies) but we have analyzed all milk samples individually; furthermore the same samples have been analyzed before and after pasteurization; ii) we have utilized a GeLC-MS analysis (semi-quantitative technique), instead of an immunohistochemical analytical techniques like the ELISA test. Additionally, there is a significant variability in previous studies concerning the retention of these proteins after HoP, which can be ascribed not only to heterogeneity in methods of human milk collection but also to treatment and storage before and after HoP [11-13].

In conclusion, donor milk needs a heat treatment for a safety storage. Holder Pasteurization is the method recommended by all international guidelines for the constitutions of Human Milk Banks. Our data are in agreement with literature showing a small decrease in the total protein content of human milk after HoP. Nonetheless, samples obtained from different donors reacted differently to heat treatment, even if processed and stored using the same conditions: in particular, 30% of the samples showed some differences in the protein profile after HoP, whereas 70% of the samples showed no detectable differences. The detectable effects on the protein profile were mainly in

colostrum samples; this variation is quite interesting from a clinical standpoint, since donor milk is mostly constituted by mature milk. The reasons for this apparent variability is not clear and would deserve further investigation. In this setting, future studies should be designed to investigate whether these differences are also confirmed by other techniques able to assess the protein changes due to thermic treatments including the interaction between proteins and sugars, or proteins and lipid with possible toxic derivatives.

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Chapter 3

The effect of Holder pasteurization on Activin A levels in human milk

CHIARA PEILA[1], ALESSANDRA COSCIA[1], ENRICO BERTINO[1], GIOVANNI LI VOLTI[2], FABIO GALVANO[2], IGNAZIO BARBAGALLO[2], GERARD HA VISSER [3] AND DIEGO GAZZOLO[4]

1Neonatology Unit, Department of Public Health and Pediatrics, University of Turin, Italy; 2Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, Catania, Italy 3Department of Obstetrics, University Medical Center, Utrecht, the Netherlands 4Department of Maternal, Fetal and Neonatal Health, C. Arrigo Children's Hospital, Alessandria, Italy

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Abstract

Introduction: There is evidence that mother's own milk is the best nutrient in terms of multiorgan protection and infection prevention. However, when maternal milk is scarce, the solution can be represented by donor milk (DM), which requires specific storage procedures such as Holder Pasteurization (HoP). HoP is not free from side effects since it is widely known that it causes qualitative/quantitative changes in milk composition, particularly in the protein content. Therefore, the aim of this study is to investigate the effects of HoP on Activin A, a neurobiomarker known to play an important role in the development and protection of the central nervous system.

Methods: In 24 mothers who delivered preterm (n = 12) and term (n = 12) healthy newborns, we conducted a pretest/test study where the milk donors acted as their own controls. Each sample was divided into two parts: the first was frozen at -80°C (Group 1); the second was Holder-pasteurized before freezing at -80°C (Group 2). Activin A was quantified using an ELISA test.

Results: Activin A was detected in all samples. There were no significant differences ($p > 0.05$) between the two groups, also when the analysis was stratified for gestational age at delivery and milk maturation degree ($p > 0.05$, for both).

Conclusion: The present findings on the absence of any side effects of HoP on the milk concentration of Activin A offer additional support to the efficacy of HoP in DM storage. Our data open up to further investigations on neurobiomarkers' assessment in human milk and their preanalytical stability according to storage procedures.

Key Words: Human milk; Donor Milk; Pasteurization Holder; Activin A; Central Nervous System

Introduction

There is ample evidence that breastfeeding is the most recommended way of promoting infant growth and development and that mother's own milk is the best nutrient in terms of multi-organ protection and infection prevention [1,2]. The explanation for this resides in the "biochemical communication" between mother and child which, thanks to human milk, allows a continuous exchange of growth factors, cytokines and hormones [3,4]. Cytokines (neuro-oxidative stress biomarkers, neurotrophic and calcium binding proteins) appear to be of particular interest for their involvement in a cascade of events leading to the correct development of the central nervous system (CNS) and cardiovascular system [5]. Although breastfeeding is considered a "life-saving" procedure, this practice is not free from issues. The main one consists of the poor availability of maternal milk, particularly in high-risk infants admitted to neonatal intensive care units (NICU). In these cases, the solution can be represented by human donor milk (DM), which nonetheless requires safe procedures for milk storage and conservation [2,6,7]. In this regard, DM can be stored at Human Milk Banks (HMB) after Holder pasteurization (HoP) (62.5°C for 30 minutes) [7]. Advantages and disadvantages associated to this procedure have been widely discussed in Literature. The advantages consist of improved protection against infections and contaminations, whilst disadvantages are represented by qualitative/quantitative changes in milk composition [6,8,9]. This may especially refer to neurobiomarkers (NB), such as Activin A, known to play a relevant role in brain development and protection [10].

Activin A is a dimeric protein belonging to the transforming growth factor beta (TGF-beta) superfamily and its receptors are widely distributed in the brain [11]. Studies in humans and animal model showed that Activin A can play a trophic and neuroprotective role on the CNS [12]. In this regard, increased Activin A levels in different biological fluids have been found in infants presenting perinatal asphyxia, prematurity and intraventricular hemorrhage [13-15]. There is evidence that this protein also exerts a neuroprotective activity (i.e., neuronal recovery, modulation in cellular and tissue growth and differentiation)[16-18]. Activin A has been also detected in human milk supporting its role as a growth factor [19]. Of note, data regarding the variation of Activin A levels in DM after HoP pasteurization are lacking.

Therefore, the purpose of the present study was to investigate the effects of HoP on Activin A concentration in healthy DM mothers.

Materials and Methods

The study protocol was approved by the local Ethic Committee of the Italian Association of Human Milk Donor Banks (AIBLUD). Mothers admitted into the study gave signed and informed consent. We conducted a pre-test/test study, where the milk donors acted as

their own controls. Milk samples (colostrum: n=12; transition: n=12; mature: n=12, respectively) [20] were collected from 24 healthy mothers having delivered at term and preterm of gestational age (GA) (GA> 37 weeks and GA< 37 weeks respectively) and coming from consecutive singleton physiological pregnancies (Table 1) [21].

We excluded mothers affected by any CNS illness; pregnancies complicated by gestational diabetes and hypertension; multiple pregnancies; fetuses with any malformation and/or chromosomal abnormalities; systemic infection; intrauterine growth retardation, or cardiac or hemolytic disease, malnutrition and maternal allergy. Standard exclusion criteria for human milk donation set forth by AIBLUD guidelines were also applied [7].

Collection and pasteurization of human milk

Fresh milk samples were collected at the same time (9-10 a.m.) into sterile, disposable, high-density polyethylene sealed bottles (Flormed, Naples, Italy). The milk was collected by means of an electric breast pump (Medela Symphony, Baar, Switzerland) with standard extraction methods. According to current guidelines and in order to collect full pumping samples, the extraction session was stopped 2 minutes after the outflow of the last drops of milk [7,22]. From the total amount of milk of each mother, a sample of 10 mL of milk was collected and then subdivided into two aliquots. The first was immediately frozen at -80°C (NO-HoP), while the second aliquot was pasteurized and then frozen at -80°C (HoP). HoP was performed with a Sterifeed pasteurizer (Medicare Colgate Ltd, Cullompton, UK) heating milk samples at 62,5° C for 30 minutes, then cooling to 10°C in approximately 20 minutes by immersion into cold water. The time interval between freezing and the analysis of the milk samples was less than 6 months (median 4 months).

Activin A measurements

Samples were immediately stored at -80°C until analysis. Activin A levels were determined using a specific ELISA test (ELH-ActivinA-1 Human Activin A ELISA) according to the manufacturer's instructions (RayBiotech, Inc.; USA). Investigators who performed the laboratory tests were blind to storage modalities. The analysis was performed within 2 hours from samples thawing. The assay detection limit is 15.00 pg/ml, the coefficient of variability intra-assay was ≤ 5.0%, and the inter-assay ≤ 10%, respectively. The assay is specific for Activin A, having been tested by the manufacturer for lack of cross-reactivity with other proteins of the Activin family.

Statistical Analysis

Demographic characteristics of maternal and neonatal outcomes were reported as mean ± SD. Activin A concentrations were expressed as median and interquartile rang-

es. Statistical analysis was performed by using two-tiled paired t-test and by Mann–Whitney two-sided U-test when data did not follow a Gaussian distribution. Comparison between groups was performed by using ANOVA one-way test for multiple comparisons. A $P<0.05$ was considered significant.

Results

Maternal and perinatal characteristics of milk donors are reported in Table 1. As expected, all mothers were in normal clinical conditions. No overt neurological injury and/or infections were observed at the sampling time points or at discharge from the hospital.

Table 1. Demographic characteristics of milk donors and infant.

Parameters	n=24
Mean (±SD) maternal age, years	35 (± 3)
Parity 1 n. (total)	13 (24)
Mode of delivery, n.	
Caesarean	6
Vaginal	18
Mean (±SD) gestational age, weeks	36 (± 5)
Mean (±SD) birth weight, g	2950 (± 1120)
Gender male (female)	11 (13)

Activin A protein was detectable in all the measured milk samples, either in term and preterm milk samples, before and after HoP. Activin A concentrations were lower in preterm milk respect term milk samples but this difference was not significant ($P>0.05$).

Table 2 Activin A concentrations (pg/mL) in human milk samples before and after HoP. Data are expressed as median and interquartile ranges.

Activin A	Before HoP			After HoP			*P*
	Median	25 centile	75 centile	Median	25 centile	75 centile	
Total milk samples	3733,5	1768,5	9379,7	3083,5	1467,2	5698,5	0.22
Colostrum	5266,0	2283,5	9291,0	3221,0	1878,5	3806,0	0.14
Transitional milk	4938,5	1948,5	11718,5	3936,0	1453,5	8598,0	0.79
Mature milk	3198,5	1708,5	9078,5	2233,05	1508,5	5638,5	0.77

As shown in Figure 1, no significant differences (P>0.05, for both) in Activin A levels were observed in all milk samples before HoP (median: 3733,5pg/mL; 25° centile: 1768,5 pg/mL and 75° centile: 9379,75 pg/mL) and after HoP (median: 3083,5 pg/mL; 25° centile: 1467,2 pg/mL and 75° centile: 5698,5pg/mL) process.

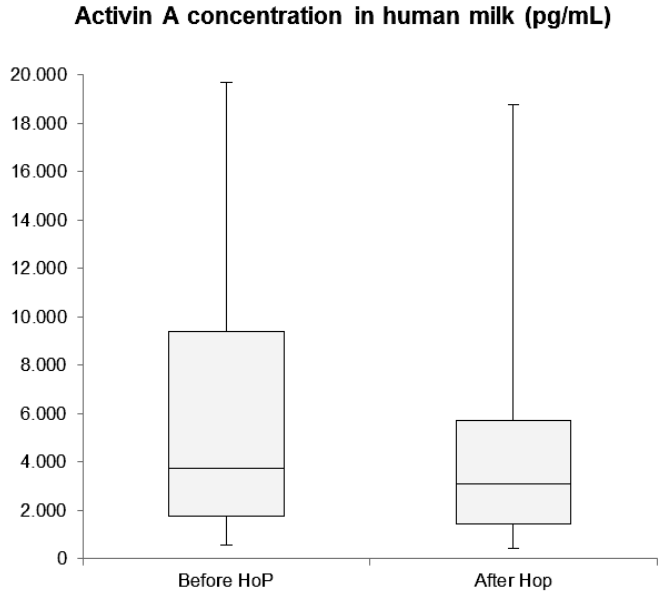

Figure 1. Activin A concentrations (pg/mL) in total human milk samples before and after HoP procedure. The lower and upper bars represent the 5th and 95th centiles, respectively; interquartile range is indicated by the box and median value is represented by the horizontal line in the box. No significant differences have been found between studied groups (P>0,05).

No significant differences (P>0.05) were found between groups when Activin A concentrations were compared in sub-groups for gestational age (i.e., term vs preterm) (Figure 2). Furthermore, Activin A levels did not differ (P>0.05) in both groups when sub-grouped based on the degree of milk maturation.

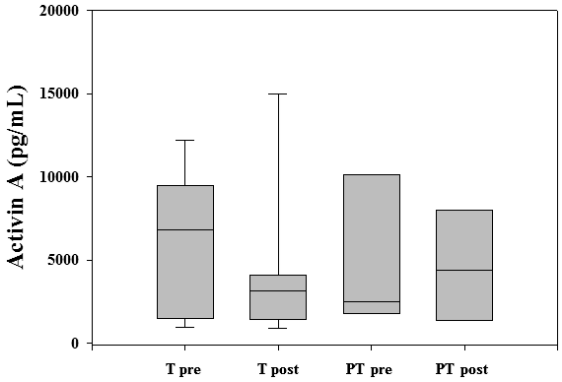

Figure 2. Activin A concentrations (pg/mL) in human milk samples before (pre) and after (post) HoP procedure in term (T) and preterm (PT) infants. The lower and upper bars represent the 5th and 95th centiles, respectively; interquartile range is indicated by the box and median value is represented by the horizontal line in the box. No significant differences have been found among studied groups (P>0,05).

Discussion

Current guidelines recommend Holder Pasteurization as the best thermal treatment for storage of donor milk in Human Milk Banks since it guarantees protection against infections and contaminations [6-8]. On the other side, there is still no conclusive consensus whether this procedure may affect the biological quality of the nutrients in donor milk [6,9].

The present study provides evidences that HoP does not modify milk concentrations of a neurotrophic protein, namely, Activin A. Indeed, no changes in protein' concentrations attributable to HoP procedure were observed throughout the various lactation phases and in correlation with gestational age. Results are, in part, in agreement with a previous observation by Luisi et al. who detected Activin A in colostrum, transition and mature milks of mothers having delivered at term of gestational age [19]. The discrepancy resides in the lack of any differences, observed in our series, among Activin A levels in mature milk versus transition and colostrum milks. The fact can be of relevance bearing in mind the changes in milk composition and properties throughout its stages of maturation [23]. Anyway, the finding of no significant changes in Activin A levels herein reported warrants further consideration. In addition, the present study identified for the first time dimeric Activin A in milk collected from lactating healthy women having delivered preterm. We observed a lower Activin A levels in this mothers groups but no significant differences were found respect the milk samples of mother having delivered at term of gestational age.

In particular, Activin A has been shown to: i) exert a neurotrophic role being involved in growth and differentiations of many CNS target cell-types [5,10]; ii) play, *in vitro* and *in vivo*, a beneficial role in recovery and survival of neurogenic cell lines and retinal

neurons decreasing ischemic brain injury [24-27]; iii) exert CNS protection from antidepressant treatment side-effects [28,29]; iv) be a trustable predictor, when measured in different biological fluids (amniotic, arterial cord blood, urine and cerebrospinal) of cerebral bleeding and damage in fetuses and newborns [5,16]. Altogether, it is reasonable to conclude that HoP *per se* guarantees an unaltered Activin A intake to the newborn and its neurotrophic properties offering additional support to the unique role of human milk. The fact is of relevance taking into account that artificial milk industrial preparation procedures have been shown to affect in a significant manner milk composition and properties [30].

Among the different Activin A functions (to date still matter of investigation and debate) it has been shown that the protein participates in a cascade of events leading to breast tissue differentiation [31,32]. Nonetheless, Activin A pro- and anti-inflammatory effects on human tissues increasing cytokine production from monocytes in normal humans peripheral blood mononuclear cells and regulating T cell development have been also reported [32-34]. The issue highlights the importance of DM administration in the immune protection of preterm infants.

Finally, there is also evidence that the protective role of Activin A is also extended to the heart tissue. In particular, it has been reported that the protein can participate in a cascade of events promoting tissue protection and regeneration in patients undergone to ischemia/reperfusion injury [35-37]. Therefore, on the basis of the aforementioned findings, it is reasonable to argue that Activin A is involved not only in the CNS development but can be considered a multi-organ trophic factor taking part to the embryogenesis. The fact that is detectable in a biological fluid at unique trophic effect such as breast milk corroborates the aforementioned hypothesis.

Lastly, bearing in mind that HoP procedures works at a temperature of 62.5°C for 30 minutes, in the present study we showed first that Activin A includes thermo-stability among its properties. This finding opens up to further investigations aimed at elucidating the protein stability under different conditions such as freezing and thawing procedures. In this respect, Lev et al. showed that storage at -80° can affect lipids and carbohydrates, whilst the total protein amount remains unchanged [38]. Finally, the present findings can be also of interest for further studies to investigate Activin A degradation during industrial processes aimed to prepare artificial milks, for which pasteurization and spray-drying procedures have been shown to affect milk composition and properties [28].

Conclusion

The present data on the absence of any side effects on Activin A milk concentration suggest the efficacy of HoP procedure in DM storage and preparation. The finding opens up to further investigations on neuro-biomarkers assessment in human milk and their pre-analytical stability according to storage procedures.

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Chapter 4

Holder pasteurization affects S100B concentrations in human milk

CHIARA PEILA[1], ALESSANDRA COSCIA[1], ENRICO BERTINO[1], LETIZIA MARRAZZO[2], FABIO GALVANO[2], GERARD HA VISSER [3] AND DIEGO GAZZOLO [4]

[1]Neonatology Unit, Department of Public Health and Pediatrics, University of Turin, Italy; [2]Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, Italy; [3]Department of Obstetrics, University Medical Center, Utrecht, the Netherlands; [4]Department of Maternal, Fetal and Neonatal Health, C. Arrigo Children's Hospital, Italy

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Abstract

Purpose. Donor milk (DM) represents an important nutrition source for high-risk newborns. Holder pasteurization (HoP) is the most recommended procedure for DM treatment, providing a good compromise between microbiological safety and biological quality. HoP was previously shown to affect DM cytokines, growth factors and hormones levels, whilst no data concerning the possible effects of HoP on neurobiomarkers (NB) are available. Therefore, our study investigated whether the concentration in DM of a well-known NB involved in brain development/damage, namely S100B, changes due to HoP.

Materials and Methods. We conducted a pretest-test study in 11 mothers, whose DM samples were sub-divided into two parts: the first was immediately frozen (-80°C); the second was pasteurized with Holder method before freezing. S100B DM levels were measured using a commercially available immunoluminometric assay.

Results. S100B protein was detected in all milk samples. Results showed significant differences between groups ($P<0.05$) in S100B levels after HoP.

Conclusions. Our data provide evidence that S100B is present in preterm milk as well as in term milk during maturation degree. Moreover, the results confirm the susceptibility of this neurotrophic factor to pasteurization stresses and the need to develop new storage techniques to preserve the biological quality of human milk.

Key Words: Central Nervous System; Donor Human Milk; Human milk; Pasteurization Holder; S100B.

Introduction

Despite technological improvement in milk-formulae milk research and industrial preparation procedures, there are still evidences on the unique composition of breast milk. This issue is of relevance especially in high risk newborn feeding in terms of multiorgan protection and infections prevention [1]. Therefore, the main target of neonatologists and biochemists points toward a wider use of breast milk in neonatal intensive care units (NICU) and to provide safer procedures for human milk storage and conservation [2]. This latter point is noteworthy since milk stored in Human Milk Banks (HMB) after pasteurization may present potential qualitative/quantitative changes in its composition [3]. Thus, the optimal pasteurization process should be able to provide as much as possible a safe and unaltered milk composition [3]. To date, Holder pasteurization (HoP) (62.5°C for 30 min) is the most studied and recommended method for the heat treatment of donor human milk (DM) [2]. HoP provides a good compromise between microbiological safety and nutritional and biological quality of the human milk. Nonetheless, HoP inactivates or reduces some immunologic and anti-infective factors [4-6] whilst data on its effects on other constituents are unknown or still matter of investigation. This especially holds for neurobiomarkers (NB) such as neurotrophic factors and calcium binding proteins, including S100B protein, known to play a relevant role in brain development [7,8].

S100B is an acidic calcium-binding protein, mainly concentrated in the central nervous system (CNS) and detectable in a variety of biological fluids including milk, where it is concentrated 200–300 fold higher than in other fluids [9,10]. Based on the protein's main known activities (neurotrophic, brain damage marker), S100B regulates different cellular functions such as growth, intercellular communication, cellular transduction signal and cellular metabolism. Bearing in mind the unique properties of breast milk, S100B has been investigated both in human and milk-formulae milks [11]. Results showed that S100B levels: i) in maternal milk were higher than formulae-milks and cow-milk [11]; ii) correlated with the degree of milk maturation [11]; iii) in formulae milks were affected by industrial preparation such as pasteurization and spray-drying procedures [12]. In this regard, data on potential effects of HoP procedure on S100B levels in human milk are still lacking.

Therefore, in the present study we investigated the effects of HoP on S100B milk concentrations from healthy mother donors.

Materials and Methods

We conducted a pre-test/test study, where the milk donors acted as their own controls. Milk samples (colostrum: n=11; transition: n=11; mature: n=11, respectively, defined

according to Playford et al.[13]) were collected from 11 healthy mothers from consecutive singleton physiological pregnancies (Table 1).

Exclusion criteria were multiple pregnancies, gestational diabetes and any maternal CNS illness; pregnancies carrying a fetus with any malformation and/or chromosomal abnormalities, systemic infection, intrauterine growth retardation, or cardiac or haemolytic disease; mothers who were tobacco smokers, drugs and alcoholic addicted; use of drugs or pharmacologically active substances; mothers who received blood transfusions or blood products, or organ transplants; perinatal asphyxia and dystocia.

The study protocol was approved by the local Ethic Committee of the Italian Association of Human Milk Donor Banks. Mothers admitted into the study gave signed and informed consent.

Collection and pasteurization of human milk

Fresh milk samples were collected at the same time-point (9-10 am) into sterile, disposable, high-density polyethylene sealed bottles (Flormed, Napoli, Italy). Milk was obtained by emptying one breast completely by means of an electric breast pump (Medela Symphony, Baar, Switzerland). From the total amount of milk of each mother, a sample of 10 mL of milk was collected and then subdivided into two aliquots. The first was immediately frozen at -80°C (NO-HoP), while the second aliquot was pasteurized and then frozen at -80°C (HoP). The HoP was performed with a Sterifeed pasteurizer (Medicare Colgate Ltd, Cullompton, UK) heating milk samples at 62,5° C for 30 minutes, then cooling to 10°C in approximately 20 minutes by immersion into cold water.

S100B measurements

Samples were immediately stored at -80°C until measurement. The S100B was measured in all samples using a commercially available immunoluminometric assay (Liaison S100, DiaSorin, Saluggia, Italy) according to the manufacturer's instructions. Investigators who performed the laboratory tests were blind to storing modalities. The assay detection limit was 0.02 μg/L, the intra-assay CV was ≤5.0%, and the inter-assay CV was ≤10%. The assay is specific for S100B, having been assessed by the manufacturer for a lack of cross reactivity with other proteins of the S100 family.

Statistical Analysis

S100B concentrations are expressed as median and interquartile ranges. Statistical analysis was performed by a comparison between groups using Mann–Whitney two-sided U-test when the data did not follow a Gaussian distribution. A $P<0.05$ was considered significant.

Results

Maternal and perinatal characteristics of the milk donors are reported in Table 1.

All mothers showed normal clinical conditions. No overt neurological injury and/or infections were observed at the sampling time-points or at discharge from the hospital.

Table 1. Demographic characteristics of milk donors.

Parameters	n=11
Mean maternal age, years	35
Parity 1 n. (total)	7 (11)
Mode of delivery, n. (%)	
Caesarean	4 (35)
Vaginal	7 (65)
Gestational Age, wks	36
Mean birth weight, g	2950
Apgar score >7 n. (total)	
At 1 min	9 (11)
At 5 min	10 (11)
Gender male (female)	5 (6)

S100B protein was detectable in all the measured milk samples. No significant differences (P>0.05, for all) have been found in S100B concentration when corrected for the degree of milk maturation. Moreover, S100B levels did not differ (P>0.05) between samples collected from mothers of preterm (median: 69.44 µg/L; 25° centile: 53.79 µg/L; 75° centile: 71,66 µg/L) and term (median: 75.55 µg/L; 25° centile: 58.83 µg/L; 75° centile: 83.70 µg/L) infants after correction for gestational age.

In Table 2 S100B levels before and after HoP procedure are reported. Significant differences in S100B concentrations were observed in all milk samples before HoP and after HoP process (P>0.005). After correction for milk maturation degree, we observed a significant reduction after HoP in transitional milk and mature milk (P>0.05) but a no significant difference in colostrum after heat treatment (P<0.05).

Table 2. S100B levels (µg/L) in milk in not pasteurized (NO-HoP) and pasteurized (HoP) groups. Data are expressed as median and interquartile ranges.

Parameters	All Samples (n=33)			Colostrum (n=11)			Transition (n=11)			Mature (n=11)		
	Median	25%	75%	Median	25%	75%	Median	25%	75%	Median	25%	75%
NO-HoP	129.50	96.30	164.0	129.50	86.10	163.0	164.00	109.37	173.75	111.0	74.72	125.75
HoP	70.00	41.60	109.0	72.80	26.40	110.0	83.40	70.00	110.50	41.60	15.92	68.9

Discussion

Nowadays, mother milk still represents the first feeding choice for all neonates. This unique nutrient is of the utmost importance especially for sick children, who require mother milk to reduce the risk of complications related to prematurity such as necrotizing enterocolitis, sepsis and bronchopulmonary dysplasia [1]. In this respect, DM was shown to represent a valid alternative [3], thus supporting the need of HMB. Of note, HMB guidelines require that DM has to be pasteurized prior to use to inactivate viral and bacterial agents [2,13,14]. Heat treatment has been reported to affect, at least in part, the nutritional and immunological properties of human milk [4-6], although no information are available to date concerning the potential effects of HoP on human milk CNS constituents.

The present study shows that HoP procedure affects the concentration in milk of a well-established CNS constituent, namely, the S100B protein. The difference in protein concentration regarded transitional and mature milks whilst no changes due to HoP procedure have been found in colostrum. Moreover, no differences among different degrees of milk maturation have been observed. The discrepancy with previous observations [9,11] may reside in different studied populations (term vs preterm-term).

S100B levels detected in milk were considerably higher when compared to those of other biological fluids [9,10]. This latter finding is in agreement with previous observations [11] and is consistent with the notion that calcium binding proteins are highly concentrated in a biological fluid such as milk in which calcium is abundant [9,10,15]. The finding of different S100B concentrations before and after HoP procedure constitutes the first observation on the effects of the pasteurization on the protein and deserves further consideration.

In particular S100B has been shown to be: i) thermostable both at room temperature and after freezing from samples collected in different biological fluids [16,17]; ii) affected, in artificial milk, by techniques in use for industrial procedures, such as spray-drying (180-185°C) [12], and iii) stable at pasteurization procedure applied in industrial procedures for treatment of milk formulae (70-72°C for 5-15 seconds) [12]. Altogether, bearing in mind that the HoP procedure consists of a heat treatment at 62.5° for 30 minutes, the possibility that medium-low temperature but for longer time than industrial procedures could affect S100B is consistent. Another explanation for the lower levels of S100B in DM may reside in the possibility that the epitopes of the protein have been modified during HoP, limiting the accuracy in the quantitative protein measurement. In addition, the possibility that HoP could also affect S100B reducing or destroying its biological activity has to be taken into the due account. The finding is of relevance since HoP has been previously shown to reduce the proteins, cytokines, growth factors and hormones content of milk [4-6]. Further studies on a wider study-population are thus needed in order to optimize CNS constituents measurement in milks as well as to improve DM components stability during treatment and storage processes.

The highest S100B levels pre-HoP and their decrease in milk concentrations following HoP warrant further consideration. The former issue offers additional support concerning the neurotrophic role of this NB [19]. In this regard, there is evidence both in humans and animal models on the trophic role of the protein in CNS fetal/neonatal development. In detail, S100B has been shown to: i) stimulate neurite outgrowth [20] trough a cascade of events in which nuclear translocation of NF-kB, up-regulation of Bcl-2 in neurons and Receptor for Advanced Glycation End Products (RAGE) are involved [21,22], ii) exert a protective action on neurons, through a RAGE-mediated effect and the activation of the Cdc42/Rac signalling pathway, under physiological (development) and pathological conditions [23,24], iii) enhance hippocampal neurogenesis, and to prevent motor neuron degeneration after sciatic nerve section [25,26], iv) be involved in the mechanisms of modulating learning and memory [27], and v) be involved (in mice pups of immunized mothers that did not express S100B protein in their milk) in morphological problems, a delay of the maturation of neurobehavioral systems and a deficit of neuromotor functions (i.e. visual abilities, somato-sensory and posture reactions, muscular strength, locomotion, and fear/orienting processes) [28]. Altogether, the evidence that milk concentration of a brain constituent involved in CNS development and protection is affected by HoP strengthens the notion on the need of further studies aimed at limiting DM depletion thus empowering its unique role [3]. This issue can be of relevance especially for the feeding of high risk newborns. Further studies on a wider study-population are needed in order to corroborate S100B transfer from gut to systemic circulation. In this regard, it has been recently observed that in the human gut, S100B protein is specifically and physiologically expressed and released in the gut by Enteric Glial Cells, morphological and functional equivalent of astrocytes and microglia in the CNS. The S100B protein can be considered as an easily diffusible pro-inflammatory cytokine which gains access to the extracellular space especially during immune inflammatory reactions. [29]

In conclusion, the present data suggest that HoP significantly modifies the concentration of S100B. This finding opens up to further investigations on NBs assessment in human milk and their pre-analytical stability according to storage procedure.

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Chapter 5

Heme oxygenase-1 in donor human milk

CHIARA PEILA[1], ALESSANDRA COSCIA[1], ENRICO BERTINO[1], GIOVANNI LI VOLTI[2], IGNAZIO BARBAGALLO[2], GERARD HA VISSER[3] AND DIEGO GAZZOLO[4]

[1]Neonatology Unit, Department of Public Health and Pediatrics, University of Turin, Italy; [2]Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, Catania, Italy; [3]Department of Obstetrics, University Medical Center, Utrecht, the Netherlands; [4]Department of Maternal, Fetal and Neonatal Health, C. Arrigo Children's Hospital, Alessandria, Italy

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Abstract

Background. When mother's own milk is not available, the best alternative is represented by donor milk (DM), i.e., human milk pasteurized with the Holder pasteurization (HoP) method in Human Milk Banks for safe storage. Advantages and disadvantages associated to this procedure have been widely discussed in Literature but currently represent the best compromise between microbiological safety and biological quality of DM.

Objective. The aim of this study is to investigate the effects of HoP on Heme oxygenase-1 (HO-1), an antioxidant protein involved in several cytoprotective actions that should play an important role in the development and protection of the gastro-enteric tract.

Methods. We collected 42 milk samples and we performed a pretest-test study where the milk donors acted as their own controls in 14 mothers (who have delivered 7 at term and 7 preterm of Gestational Age). Milk samples were divided into two parts: the first was frozen (-80°C); the second was Holder-pasteurized before freezing (-80°C). HO-1 was quantified using an ELISA test.

Results. HO-1 was detected in all samples. There were no significant differences in HO-1 concentrations between term and preterm milk samples ($P>0.05$). Likewise, no significant differences in HO-1 content were found between raw and pasteurized milk samples ($P>0.05$). There were no significant differences when studied groups were corrected for milk maturation degree ($P>0.05$).

Conclusion. The data suggest that HO-1 does not significantly differ between preterm and term milk samples and that HoP does not affect HO-1 concentration in Human Milk.

Key Words: Human milk; Donor Human Milk; Pasteurization Holder; Hemeoxygenase-1; Nutrition of preterm infants; NEC.

Background

In literature, there are several evidences of the unique role of human milk for infant nutrition [1,2]. Feeding preterm and term infants with human milk is associated with significant long-term beneficial effects such as reduced risk of cardiovascular diseases, diabetes, obesity and cancer [1,3]. During the first months of life, short-term benefits are also present, such as supporting the development of the immune system and providing protection against infectious diseases [1]. When mother's own milk is not available, the best alternative is represented by donor milk (DM), i.e., human milk pasteurized with the Holder pasteurization (HoP) method in Human Milk Banks (HMBs) for safe storage [4,5]. The available literature confirms the persistence of some of the benefits of human milk also in donor milk, especially regarding the protection against Necrotising Enterocolitis (NEC) for preterm newborns [6-9]. The short term benefit of HM and DM is supported probably not only by secretory immunoglobulin A but also by other immunologically active components such as antimicrobial factors, cytokines, chemokines and growth factors [10-13]. Additionally, the antioxidant properties attributed to HM play a role of paramount interest, although the underlying mechanisms are still unclear [14,15]. The explanation may reside in the presence of antioxidant enzymes, an important group of which is constituted by heat shock proteins (HSPs), whose presence in biological fluids has been associated to the capacity of binding to specific cell surface receptors such as CD91 [16]. Among HSPs, heme oxygenase-1 (HO-1 or HSP32) is a stress-inducible rate-limiting enzyme in heme-catabolism which is associated to a strong cytoprotective effect [17] thanks to its multiple catalytic by-products. HO-1 has recently been detected in different biological fluids (blood and cerebrospinal fluid), where its presence is reasonably related to its putative protective action [18,19]. Although the potentially important role of HO-1 in human milk, only one previous study has investigated the concentration of HO-1 in the HM of healthy mothers having delivered at term of Gestational Age.

The present study will evaluate (a) the pattern of HO-1 in preterm and term human milk, over the transition from colostrum to mature milk; (b) the concentration of HO-1 in donor milk, to evaluate the possible effect of HoP on this protein.

Methods

We performed a pre-test/test study, where milk donors acted as their own controls. Mothers admitted into the study gave signed and informed consent. The study protocol was approved by the local Ethic Committee of the Italian Association of Human Milk Donor Banks (AIBLUD).

We enrolled 14 healthy mothers: 7 who have delivered at term (Gestational Age >37 weeks) and 7 who have delivered preterm (Gestational Age <37 weeks) [20]. Samples

were collected from colostrum 48h after birth, from transition milk on Days 7 and 14, and from mature milk on Day 30 after birth (colostrum: n=14; transition: n=14; mature: n=14, respectively) [21]. Standard exclusion criteria for human milk donation set forth by AIBLUD guidelines were applied [4]. Moreover we excluded also: multiple pregnancies, any CNS illness, gestational diabetes and hypertension, systemic infection, intrauterine growth retardation, cardiac or metabolic or hemolytic disease, malnutrition and maternal allergy and fetuses with any malformation and/or chromosomal abnormalities.

Collection and pasteurization of human milk

Fresh milk samples were collected at the same time (9-10 a.m.) into sterile, disposable, high-density polyethylene sealed bottles (Flormed, Naples, Italy). Milk was collected with standard extraction methods by means of an electric breast pump (Medela Symphony, Baar, Switzerland). According to current guidelines and in order to collect full pumping samples, the extraction session was stopped 2 minutes after the outflow of the last drops of milk [4,22]. From the total amount of milk of each mother, a sample of 10 mL of milk was collected and then further divided into two parts. The first was immediately frozen at -80°C (NO-HoP), while the second part was pasteurized and then frozen at -80°C (HoP).

HoP was performed with a Sterifeed pasteurizer (Medicare Colgate Ltd, Cullompton, UK) heating milk samples at 62,5° C for 30 minutes, then cooling to 10°C in approximately 20 minutes by immersion into cold water.

Heme oxygenase-1 measurements

Samples were immediately stored at -80°C until analysis. Heme oxygenase-1 levels were determined using a specific ELISA test (SL0838HU-97 Human heme oxygenase 1 ELISA) according to the manufacturer's instructions (SungLong Biotech Co., Ltd.; China). Investigators who performed the laboratory tests were blind to storage modalities. The assay detection limit is 1.00 ng/ml, the coefficient of variability intra-assay is ≤ 5.0%, and the coefficient of variability inter-assay is ≤ 10%. The assay is specific for Heme oxygenase-1.

Statistical Analysis

Demographic characteristics of maternal and neonatal outcomes were reported as mean ± SD. HO-1 concentrations were expressed as median and interquartile ranges. Statistical analysis was performed by using two-tiled paired t-test and by Mann–Whitney two-sided U-test when data did not follow a Gaussian distribution. Comparison between groups was performed by using ANOVA one-way test. A $P<0.05$ was considered significant.

Results

Maternal and perinatal characteristics of milk donors are reported in Table 1.

Table 1. Demographic characteristics of milk donors.

Parameter	n=14
Mean (±SD) maternal age, years	35±5
Parity 1 n. (total)	10 (14)
Mode of delivery, n.	
Caesarean	5
Vaginal	9
Mean (±SD) gestational age, weeks	36±4
Mean (±SD) birth weight, g	2950±1100
Gender male (female)	6 (8)

HO-1 was detectable in all human milk samples. Its concentration was higher in colostrum (median 74,8 ng/ml; 25° centile 59,1 ng/ml and 75° centile 83,7ng/ml) with respect to transition milk (median 64,2 ng/ml; 25° centile 52,9 ng/ml and 75° centile 77,7 ng/ml) and to mature milk (median 72,2 ng/ml; 25° centile 71,4 ng/ml and 75° centile 86,6 ng/ml): nonetheless, the differences in HO-1 concentration were not significant, as represented in Figure 1 (P>0.05).

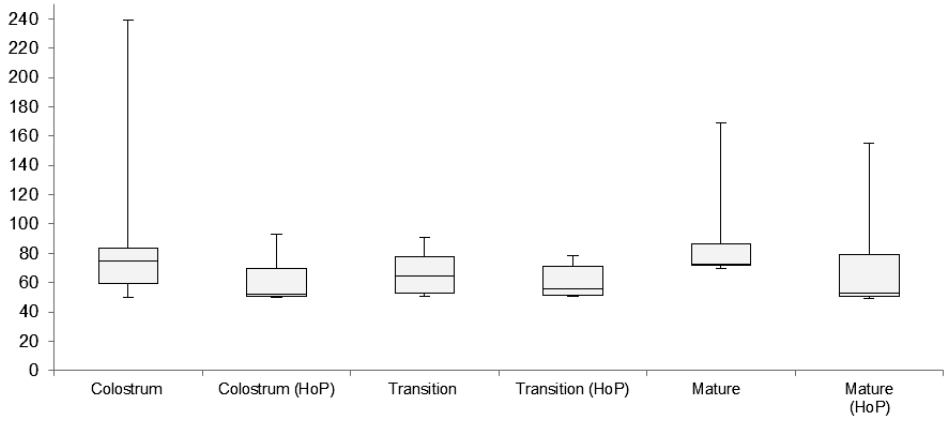

Figure 1: Heme oxygenase-1 concentrations (ng/mL) in milk samples during maturation degree, before and after HoP. The lower and upper bars represent the 5° centile and 95° centile, respectively; interquartile range is indicated by the box and median value is represented by the horizontal line in the box . No significant differences have been found between studied groups (P>0,05).

After correction for gestational age, we can conclude that HO-1 does not significantly differ between preterm (median 69,4ng/ml; 25° centile 53,7ng/ml and 75° centile 71,6ng/ml) and term milk samples (median 75,5ng/ml; 25° centile 58,8ng/ml and 75° centile 83,7ng/ml), P>0.05 (Figure 2).

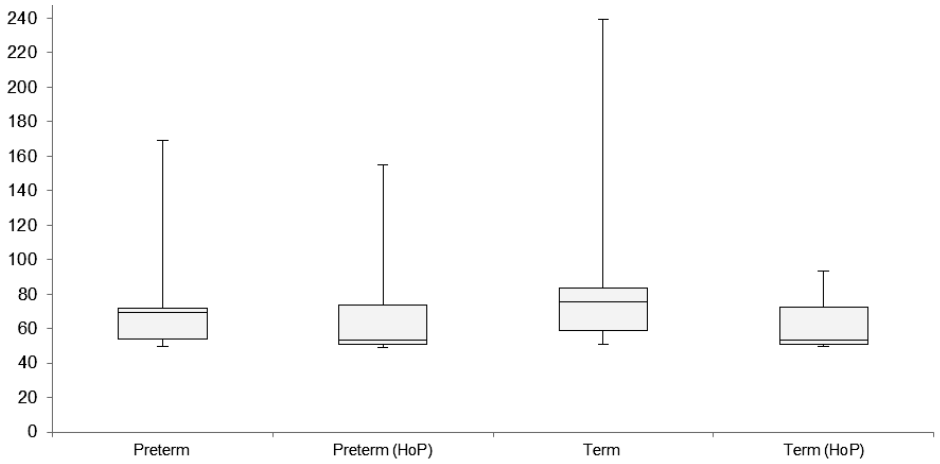

Figure 2: Heme oxygenase-1 concentrations (ng/mL) in preterm and term samples, before and after HoP. The lower and upper bars represent the 5° centile and 95° centile, respectively; interquartile range is indicated by the box and median value is represented by the horizontal line in the box . No significant differences have been found between studied groups (P>0,05).

HO-1 was detectable also in all pasteurized milk samples, as shown in Figure 1. No significant differences in HO-1 levels were observed in all milk samples before HoP (median 71,4ng/ml; 25° centile 57,0ng/ml and 75° centile 83,5ng/ml) and after HoP (median 53,7ng/ml; 25° centile 50,7ng/ml and 75° centile 73,8ng/ml) (P>0.05). No significant differences (P>0.05) have been found between groups when HO-1 concentrations were compared sub-grouping for gestational age (term vs preterm) (Figure 2). Furthermore, HO-1 levels did not differ (P>0.05, for both) in both groups when compared for the degree of milk maturation (Figure 1).

Discussion

Human milk contains several biological factors that are involved in a newborn's growth and immune system regulation, and which are fundamental to ensure protection against infection and to prevent NEC in preterm newborns [1,23]. Among these factors, HO-1 is an antioxidant protein and an enzyme of the Heat Shock Protein group which has been associated to important cytoprotective actions [23-27].

Our study is the first to show that HO-1 is present in preterm human milk and in donor milk. No statistically significant differences were found between preterm and term milk samples; likewise, our results showed a not significant decrease in HO-1 levels as the milk matures, in both term and preterm milk. These findings are in agreement with the notion that the concentrations of colostrum and milk constituents change with suckling time. Only one study was conducted previously in order to evaluate the presence of HO-1 in term human milk [28]. Our data are in agreement with the results of Li Volti et al., although we did not find significant differences between colostrum and mature milk [28]. The presence of a typical intracellular protein such as HO-1 in milk is consistent with previous observations demonstrating the presence of other intracellular proteins (e.g., α-lactalbumin, calmodulin and osteocalcin) in extracellular space and/or biological fluids. The presence of such proteins in milk is related to an active secretory mechanism and/or to the anatomical and physiological characteristics of the mammary gland (i.e., apocrine mechanism of secretion).

Moreover our study is the first that evaluates the effect of HoP on HO-1 levels in human milk. The data show that this heat treatment does not affect significantly HO-1 concentrations also after correction for gestational age and maturation degree of the human milk. Currently, Holder pasteurization (62.5°C for 30 minutes) is the most studied and recommended method for the heat treatment of donor human milk in Human Milk Banks, since it represents a good compromise between microbiological safety and nutritional and biological quality of the human milk [4,5]. In fact, this method is able to inactivate milk pathogens but partially degrades immunologic and anti-infective factors such as IgA, IgG, lysozyme, lactoferrin, lymphocytes, growth factors and lipase as well [29,30]. Nonetheless, with Holder pasteurization other key nutritional factors (i.e., oligosaccharides, lactose, LCPUFA, fatty acids and vitamins) remain unaffected [29,31]. The loss of biological factors through pasteurization may have significant implications and this is particularly important in preterm infants with an immature immune system, who are at increased risk of developing necrotizing enterocolitis (NEC).

In particular milk HO-1, similarly to other milk antioxidant enzymes, may exert protection via its antioxidant activity, which is related to the conversion of free heme into three end products: (i) biliverdin, which is rapidly reduced into bilirubin (which itself possesses antioxidant activity) [32]; (ii) carbon monoxide, a potential gaseous neurotransmitter and vasodilator with anti-inflammatory and antiapoptotic activities [17]; and (iii) iron (Fe^{2+}), which is sequestered by iron-binding proteins [33,34]. Moreover HO-1 has probably an immunoregulatory role that is not only dependent on its enzymatic activity but is related to its ability to bind specific receptors, thus regulating the immune response. In fact extracellular stress proteins, including HSP, are emerging as important mediators of intercellular signaling and transport [35,36]. Release of such proteins from cells is triggered by behavioral stress, as well as by exposure to immunological “danger signals” [34]. After release into extracellular fluid, HSP may then bind to the surfaces of adjacent cells and initiate signal transduction cascades, as well as transport of cargo

molecules such as antigenic peptides and chaperokines with an immunomodulatory effect [16]. In particular, Li Volti et al., using a molecular modeling approach, found that an important immunoregulatory receptor (i.e., CD91) is the possible ligands of HO-1. Nevertheless, integrating experimental data with in silico data also highlighted the putative role for HO-1 as an immunomodulatory molecule [28].

Bearing in mind the different functions of HO-1 in several tissue, the data reported in literature by four meta-analysis showing a reduction in NEC incidence in preterm babies fed with donor milk compared to those fed with preterm formula [6-9], and the unclear pathophysiology of NEC (immature gastrointestinal epithelium, impaired immunological defences, enteral feeding and bacterial colonization), it is possible to argue that human milk HO-1 may play a role in development and regulation of immune system of the gastroenteric tract.

Conclusion

Our study showed that HO-1 is present not only in term human milk but also in preterm human milk in similar concentrations. Additionally, our data suggest that the biological value of human milk associated with the HO-1 content is maintained after Holder pasteurization. Further data concerning the metabolic fate of HO-1 in the gastro-enteric tract is needed to corroborate the hypothesis that HO-1 participates in the nutritional and immunoregulatory effects of milk.

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Chapter 6

Human milk Adrenomedullin is unstable during cold storage at 4°c

CHIARA PEILA[1], ALESSANDRA COSCIA[1], ENRICO BERTINO[1], GIOVANNI LIVOLTI[2], IGNAZIO BARBAGALLO[2]AND DIEGO GAZZOLO[3]

[1]Neonatology Unit, Department of Public Health and Pediatrics, University of Turin, Italy; [2]Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, Catania, Italy; [3]Department of Maternal, Fetal and Neonatal Health, C. Arrigo Children's Hospital, Alessandria, Italy

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Abstract

Introduction. Under some circumstances Human Milk (HM) extraction and refrigerated storage may be necessary. Depending on the length and on the type of cold storage, milk may lose some important properties but current advices on safe HM storage are discordant. Moreover until now no data in literature was present on the effect of prolonged cold storage on biological active components of the HM such as Adrenomedullin (AM). This important peptide is involved in response to hypoxia and inflammation, associated with neovascularization, in several tissues. The aim is evaluate: (a) the presence of AM in preterm and term human milk; (b) the concentration of AM in refrigerated milk at 4°C at 24h intervals, up to 96 hours of storage.

Materials and Methods. The experiment was repeated 4 times. Immediately after collection, each HM sample deriving from each mother was divided into two parts: "Pool" line and "Single Mother" line. One part (Pool line) was pooled and then divided into 5 aliquots. The other part (Single Mother line) was divided into 5 aliquots. From each line, one aliquot, was analyzed within 3 hours, while the others were stored in the refrigerator for 24, 48, 72 and 96 hours, respectively and then analyzed. AM levels were determined using a specific ELISA test.

Results. AM was detectable in all samples. Its concentration was significant higher in preterm milk with respect to term milk ($P<0.05$). Significant differences were observed during the cold storage: the AM levels decreased steadily during the storage and the remaining concentration at 96 hours is approximately 2%.

Discussion. This study provides evidences regarding the presence of AM in HM, regardless of the gestational age. In particular, the refrigeration of fresh human milk in controlled conditions significantly affected its bioactivity and nutritional quality related with AM, already at 24 hours.

Key Words: Adrenomedullin; refrigeration; cold storage; human milk, nutrition.

Background

Breastfeeding is the gold standard nutrition for infants [1]. However, breastfeeding is not always possible and, under some circumstances (mainly in Neonatal Intensive Care Units), HM extraction and refrigerated storage may be necessary. Moreover, the storage of refrigerated milk may also be performed at home for milk to be donated to milk banks, or for later use by the mother's own infant. Depending on the length and on the type of cold storage, HM may lose some important nutritional and functional properties. Current advices on safe HM storage are discordant [2-4] due to the variability of the findings reported in the literature [5]. Recent studies highlighted that the total protein count and the protein profile were not affected by refrigerated storage at 4°C for 96 hours and Giribaldi et al. observed that the refrigeration process did not affect the abundance of important nutritional markers such as the available Lysine, sIgA, lactoferrin and Lysozyme content [6,7]. On the other hand, until now, no data in literature was present concerning the effect of prolonged cold storage on several biological active components of the HM.

Adrenomedullin (AM) is a C-amidated peptide, involved in response to hypoxia and inflammation, which are associated also with neovascularization. Recent studies indicate that AM is synthesized also in the mammary gland and secreted in breast milk [8], although the data are discordant on its concentration in milk of mothers having delivered at term of the gestational age. On the other hand the presence of AM in HM and the variation in its concentration between the different milk maturation degrees and between healthy and pathological mothers suggest that AM may have an important role in the regulation of growth and maturation of the neonatal gastrointestinal tract [9-11].

In order to elucidate some of the uncertainties regarding AM in human milk, we evaluated: (a) the presence of AM in term and also in preterm human milk; (b) the effect on AM concentration in refrigerated milk at 4°C at 24h intervals, up to 96 hours of storage.

Methods

Sample collection

HM samples were collected at the Neonatal Intensive Care Unit (NICU) of the University of Turin, from healthy mothers having delivered preterm and at term of gestational age. Standard exclusion criteria for human milk donation set forth by AIBLUD guidelines were also applied [3]. Mothers admitted into the study gave signed and informed consent. The study protocol was approved by the local Ethic Committee of the Italian Association of Human Milk Donor Banks (AIBLUD). The experiment was repeated 4 times to ensure reproducibility of the results, we enrolled 3 mothers for each time (total donat-

ing healthy mothers: 12). Fresh milk samples were collected at the same time (9-10 a.m.) into sterile, disposable, high-density polyethylene sealed bottles (Flormed, Naples, Italy). Milk was collected with standard extraction methods by means of an electric breast pump (Medela Symphony, Baar, Switzerland). Immediately after collection, each HM sample deriving from each mother was divided into two parts: "Pool" line and "Single Mother" line. The "Pool" line part was pooled in a sterile bottle with the other "Pool" line parts deriving from the other 2 mothers (total volume: 50 ml) and then divided into 5 aliquots. The "Single Mother" line part was immediately divided into 5 aliquots. From each line, one aliquot, (0h) was immediately frozen at -80°C until the analysis. The others aliquots (24h, 48h, 72h, 96h) were stored in the refrigerator at 4°C in the NICU respectively for 24, 48, 72 and 96 hours, and then immediately frozen at -80°C until the analysis . The replicate pools were kept separated and analyzed independently. The temperature of the refrigerator was constantly monitored by two mini data loggers equipped with internal probes (Testo 174T, Lenzkirch, Germany) placed on the bottom and top shelves of the NICU fridge, and programmed to record the temperature every 5 minutes.

Adrenomedullin analysis

Adrenomedullin levels were determined using a specific ELISA test (SL007HU- Human adrenomedullin -ADM- ELISA KIT) according to the manufacturer's instructions (SungLong Biotech Co.Ltd.;China). Investigators who performed the laboratory tests were blind to storage modalities. The assay detection limit is 0.05 ng/L, the assay range is 2 ng/L-40 ng/L. The assay is specific for Human AM.

Statistical analysis

Demographic characteristics of maternal and neonatal outcomes were reported as mean ± SD. Adrenomedullin concentrations were expressed as mean ± SD. Statistical analysis was performed by using two-tiled paired t-test and by Mann–Whitney two-sided U-test when data did not follow a Gaussian distribution. Comparison between groups was performed by using ANOVA one-way test for multiple comparisons. A $P<0.05$ was considered significant.

Results

Demographic Characteristics

The 12 donor mothers were healthy women having delivered; 6 at term (Mean gestational age at birth: 39+/- 1weeks; mean birth weight: 3010+/- 220g) and 6 preterm (mean gestational age at birth: 30 +/- 1weeks; mean birth weight: 1650 +/- 140g). 8

mothers delivered by Vaginal and 4 by Cesarean Section. 5 newborns were males and 7 females. The mean date of delivery was one month before the donations.

Adrenomedullin levels

Adrenomedullin was detectable in all human milk samples. Its concentration was higher in preterm milk (mean: 200,0 ng/L; SD +/- 189,9 ng/L) with respect to term milk (mean 18,1 ng/L; SD +/-10.9 ng/L) and the differences in concentration were significant (P<0.05). AM was detectable also in all refrigerated milk samples, Table 1.

Table 1 Adrenomedullin concentrations (ng/L) in human milk during the cold storage. Data are expressed as mean and SD.

AM	0h	24h	48h	72h	96h
TS	272.8 ±195.3	120.1 ± 140.3	20.55 ± 6.3	13.9 ± 6.8	7.3 ±4.8
PL	341.4 ±132.5	234.3 ± 178.7	23.3 ± 2.1	12.4 ± 8.3	7.3 ±6.2
SML	242.3 ±217.4	69.4 ± 90.2	19.3 ± 7.2	14.5 ± 6.6	7.3 ±4.5

Abbreviations: Total sample, TS; pool line, PL; single mother line, SML.

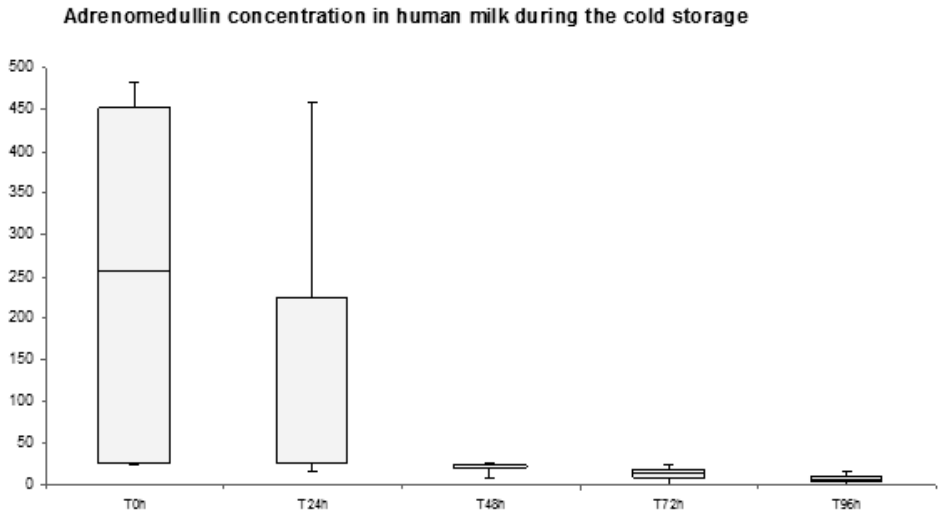

Figure 1 Adrenomedullin concentrations (ng/L) in total human milk samples during the cold storage at 4°C for 96 hours. The lower and upper bars represent the 5th and 95th centiles, respectively; interquartile range is indicated by the box and median value is represented by the horizontal line in the box.
* Significant differences between T0h and the other time points (P<0,05).

As shown in Figure 1, significant differences in AM levels were observed during the cold storage. The data show a significant reduction of 56% in concentrations in all samples at 24 hours. The AM levels decreased steadily during the storage and the remaining concentration at 96 hours is approximately 2%. This trend of reduction is similar in the

"Pool" line and in the "Single mother" line. Indeed, a significant difference ($P<0.05$) was found also when AM concentrations in milk samples were corrected for gestational age.

Discussion

The biologically active components found in human milk participate in multiple physiological processes including modulation of gastrointestinal functions, microbial growth control and immunoregulation [12]. Cold storage of human milk is a common practice both in hospitals and at home, but the effect on these components is already unclear. The maximum refrigeration time for human milk ranges between 24 hours and 8 days, according to current advices. Such variability reflects the heterogeneity of the scientific sources, which can be attributed to differences in the study design and in the methodological approaches [5]. Recent studies concluded that human milk can be stored for 96h at 4°C without affecting the overall milk integrity but several important biological compounds were not considered at all [6,7].

Our study shows that prolonged refrigeration of HM at 4°C affects its original content of adrenomedullin. This protein is not thermostable at 4°C: AM is significant reduced (56%) at 24h and is nearly undetectable at 96h. We performed our analysis not only in HM pool but also in HM samples deriving from the single mothers in order to compare the results on two different modality of storage of HM (Human milk banks and home, respectively). Our data shown that the model of AM decrease is similar in both lines. Moreover we related the decrease content of the AM only at prolonged refrigerated storage because the protein stability, under different conditions such as freezing and thawing procedures, are certain. In this respect, Lev et al. [13] showed that the total protein amount remains unchanged during the storage at -80°c; data confirmed by Ahrabi et al. regarding the freezer storage, at -20°C, of human milk for up to 9 months for refrigerated milk [14]. The thawing procedures were investigated recently by Handa et al. [15] and no changes in protein were observed between processing methods of thawing and warming of human milk.

We detected AM in all HM samples collected and these data are in agreement with the literature. Before the discovery of the presence of AM in mouse milk [8], 3 previous studies have evaluated the presence of the AM in human milk. Pio et al. and Ohta et al. focused their studies in human milk of healthy mothers having delivered at term and obtained discordant results on AM concentrations [9-11]. Ohta et. al detected AM only in 21% of samples (assay system IRMA) vs results of Pio et al., who found AM in all milk samples (radioimmunoasssay RIA). Cekmen et al. analyzed HM of healthy and preeclamptic women and found AM in all samples (HPLC) [9-11].

Our data show for the first time the presence of this important peptide also in HM of mother having delivered preterm infants and demonstrate that AM levels in preterm milk are significantly higher in respect to the term milk. Our results are in agreement

with the previous literature demonstrating that AM levels in HM are higher than those reported in plasma: several human hormones and growth factors present in milk exceed their plasma concentration [16]. Anyway, the finding of significant changes in AM levels herein reported warrants further considerations. AM is a regulatory peptide and its expression was demonstrated in several tissues and biologic fluids such as plasma, cerebro-spinal fluid, sweat, amniotic fluid and urine [17-19]. AM has been implicated in the modulation of several physiological functions including cardiovascular tone [20], central brain activity [21-24], bronchodilation [25], renal function [26], hormone secretion [27], cell growth-differentiation [28] and immune response [29,30]. Moreover AM has been investigated for its involvement in ischemia-reperfusion injury whilst in healthy infants has been shown to participate in the cascade of events promoting fetal/neonatal cardiovascular adaptation [22-24]. AM has been also assessed for evaluation of beneficial/side-effects of in-utero vasodilation therapeutic strategies in pregnancies complicated by fetal chronic hypoxia [22-24]. In this regard, an observation on AM and the occurrence of adverse neurological outcome has been reported in infants with congenital heart disease [22-24]. Bearing in mind these important functions, it is possible to speculate that the presence of the active peptide AM in HM, and their variability in concentrations between milk degree (from colostrum to mature milk), gestational pathologies and gestational age of delivery, could have some direct impact in the development of the neonate due to the several physiological activities that have been associated to it. In relation with the gastrointestinal tract, immunoreactive AM has been detected in human stomach, duodenum, jejunum, ileum and colon [31], and specific binding sites have been detected in rat stomach [32]. This distribution suggests a role for AM in the regulation of secretory-motor functions in the gastrointestinal tract, as well as in its development during the embryogenesis and the period immediately following birth. Since the developing intestine in the neonate is considered to be one of the main target organs for the growth factors present in human milk, Pio et al. [9] demonstrated that milk has a growth-promoting activity on human small intestinal epithelial cell line (Int-407). These authors suggest that since MoAb-G6 partially blocks the milk-induced growth, AM may be one of the growth factors present in milk. AM has also been described as an agent with antimicrobial activity against gastrointestinal microorganisms [29,30]. This activity could be important for the protection of the neonate against gastroenteritis produced by intestinal pathogens. In the and, since some peptides are absorbed from the neonatal gastrointestinal tract and appear intact in plasma, [33] AM could also exert an activity in the modulation of tissue growth as well as in the regulation of the immune system.

Finally, the present findings can also be of interest for future studies aimed at investigating the variability of AM concentrations in colostrum/transitional/mature milk under the same storage conditions and in the different gestational age of delivery. Moreover, it would be interesting to correlate the AM milk levels with the following parame-

ters: AM plasma levels in mothers, frequency of breastfeeding, type of delivery, number of births and also with gestational pathologies.

Conclusion

In conclusion, this study provides new and incremental evidences regarding the presence of adrenomedulin in human milk, regardless of the gestational age at delivery. In particular, refrigeration of fresh human milk in controlled conditions for 96 hours significantly affected its bioactivity and nutritional quality related with adrenomedullin.

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Chapter 7

Summary & conclusions

Summary and Discussion

Recently, several studies have emphasized that fresh own mother's breast milk is the first choice in preterm infant feeding and that strong efforts should be made to promote lactation [1]. For this reason, it has been the goal of international neonatal networks to increase breastfeeding rates at discharge from the NICU above the threshold of >20%. Nonetheless, when mother's own milk is not available, DM is highly recommended [1]. DM should be provided from an established HMB, which has to follow specific safety guidelines [2]. The presence of a HMB does not decrease breast-feeding rates at discharge, but decreases the use of formula during the first weeks of life [3].

Among the reported evidence of the benefits in preterm newborns deriving from the use of DM, protection against NEC is particularly important and should be highlighted [4]. The potential advantages of unfortified DM on improved feeding tolerance and a reduced cardiovascular risk during adolescence are still a matter of debate [3].

Storage and processing of HM reduce some of its biological components, which may lose, in part, some of their health benefits. From a nutritional point of view, DM (like HM) is constantly subject of research since new molecules involved in developmental processes have been identified, such as the *"trophic factors"* [5].

Future research should focus on improvements of milk processing in HMB (particularly heat treatment) and on further evaluation of the potential clinical benefits of processed and fortified DM.

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In Chapter 1 we have provided an overview of the main findings related to the effects of HoP on the biological and nutritive components of DM and have shown a high variability among literature data. Apparently it is very difficult to quantify the actual effects of HoP on the biological and nutritional properties of the milk. Substantial discrepancies exist not only between the protocols applied in different studies but also between the standard operating procedures adopted by HMBs and the experimental methods reported in research protocols. When pasteurization is performed for research purposes, it is usually carried out with laboratory-scale equipment, which is not equivalent to the pasteurization environment used in HMBs [6,7]. Moreover, samples submitted to thermal treatment in laboratories often consist of a few milliliters of milk (typically less than 10 mL) [7,8], whereas HMBs routinely use industrial pasteurizers, specifically designed and validated for the pasteurization of larger quantities of human milk (up to 200 mL of milk for each container). Another limitation is the fact that several studies do not provide precise information concerning the equipment used and the pasteurization parameters applied in the study (e.g., time, temperature, and duration of the different phases of the processing) [9,10]. Furthermore, conditions applied in research protocols for milk expression, handling, processing and storage before analysis are extremely variable, whereas storage conditions in HMBs are standardized and well defined by written pro-

tocols [6-13]. Based on these considerations, it is not surprising that significant differences are present when comparing results obtained in clinical practice and research conditions. Thus, it remains a matter of debate whether the studies available in literature adequately describe the impact of HoP on milk properties and if the nutritional qualities of milk are preserved after pasteurization.

Altogether, the available data show that HoP affects several HM components, even though it is rather difficult to quantify the degradation level. Additionally, it appears that some biochemical patterns have been investigated more extensively than others, whereas some milk components were not considered at all. Proteins are more significantly affected by HoP but the results concerning specific biologically active molecules (such as cytokines and growth factors) remain uncertain, due to the vast number of different compounds analyzed in each study and to the paucity of comparable results.

Our studies were designed to investigate HoP adapting the protocols to match the actual procedures of storage, handling and processing of donor milk practiced by HMBs, to provide results relevant for the actual clinical management of DM.

In **Chapter 2** we evaluated the effect of HoP on the protein profile using a semi-quantitative GeLC-MS analysis technique. GeLC-MS was chosen as it is particularly well-suited to evaluate the complex array of biologically active proteins composing the HM protein profile. Samples obtained from different donors reacted differently to heat treatment although processed and stored at the same conditions. Briefly, about 30% of the samples analyzed showed differences in the protein profile after HoP, whereas the remaining 70% did not. The main detectable protein profile changes were observed in colostrum, thus providing further support to the use of DM since no evident changes were shown in mature milk. These findings deserve further investigation. Specifically, the main open question concerns the interactions between proteins and sugars or lipids due to thermic treatments, with the possible generation of inactive (or even toxic) derivatives.

In **Chapter 3** we investigated the effects of HoP on the DM concentration of a well-established biomarker of CNS development/damage, namely Activin A. Results showed that HoP does not affect Activin A concentrations in DM at different milk maturation degrees. Furthermore, the present study showed first the presence of Activin A in milk collected from preterm deliveries. The loss of biological factors through pasteurization may have significant implications; this is particularly important in preterm infants with an immature immune system, who are at increased risk of developing NEC. The evidence that HoP guarantees an unaltered Activin A intake to the newborn further confirms the benefits of feeding preterm newborns with DM as compared to artificial milk [14]. This is of relevance considering that artificial milk industrial preparation procedures have been shown to affect to a significant extent both the milk composition and properties [15]. Moreover, an unaffected Activin A concentration in DM is important since on the basis of several studies it is reasonable to assume that Activin A is involved

in embryogenesis and exerts a unique trophic effect in biological fluids [16]. In fact, Activin A, its receptors and binding proteins are widely distributed throughout the brain [16]. Studies in experimental models and in case of acute brain injury in the human strongly correlate enhanced Activin A expression as a common response to acute neuronal damage of various origins. Even interesting is the finding that Activin A is able to support the survival of neurogenic cell lines and neurons and to offer protection against neurotoxic damage [17]. Moreover up-regulation of this neurotrophic factor by antidepressant treatment and atrophy of limbic brain regions in response to stress or in depressed patients has resulted in a neurotrophic hypothesis of depression [18]

HM is believed to contain biological factors involved in the regulation of newborn growth, including brain development. Moreover the presence of S100B, a calcium-binding protein in a biological fluid such as milk, in which calcium is abundant, is not surprising in the light of the consideration that other calcium-binding proteins (e.g. alpha-lactalbumin, calmodulin, osteocalcin) have already been detected in milk [19-21].

Therefore, in **Chapter 4** we investigated whether HoP procedure could somehow affect the neurotrophic S100B protein. Results showed an "unexpected" susceptibility of the protein to HoP, especially in transitional and mature milks, whereas no differences were detectable in colostrum. These finding deserve further consideration. The available data confirm that i) S100B collected from different biological fluids is stable both at low and room temperature for long time [19,22], ii) industrial pasteurization procedures for treatment of milk formulae (70-72°C for 5-15 seconds) do not affect S100B [15], and iii) S100B is affected by industrial spray-drying techniques (180-185°C) [15]. Overall it is possible to conclude that temperature *per se* does not affect S100B characteristics. Nonetheless, bearing in mind that the HoP procedure consists of a heat treatment at 62.5° for 30 minutes, the possibility that medium-low temperature for longer time could affect S100B is quite well possible. Another explanation for the lower levels of S100B in DM may reside in the possibility that the epitopes of the protein are modified during HoP, limiting the accuracy in the quantitative protein measurement. In addition, the possibility that HoP could also affect S100B by reducing or destroying its biological activity has to be taken into account.

In **Chapter 5** we studied the effects of HoP on heat shock proteins (HSPs) such as HO-1. Among the several functions of HM, antioxidant properties have also been attributed [23]: the explanation may reside in the presence of antioxidant enzymes and HSPs that have been related to the capacity of binding to specific cell surface receptors such as CD91 [24]. CD91 is an important immunoregulatory receptor that has been previously identified as a receptor for the serum protein α2-macroglobulin (a 'natural' protease inhibitor) and then as a common receptor for all identified HSPs [25,26]. An increasing body of evidence suggests that CD91 represents an important route for stimulating a CD8+ T-cell response by major-histocompatibility-complex-class-I-restricted presenta-

tion. This may also be a fairly general mechanism by which the innate immune system may stimulate the adaptive arm in viral infections and tumors [27].

Among HSPs, HO-1 is the rate-limiting enzyme in heme catabolism that is associated with strong protective effects [28]. The presence of HO-1 has been recently detected in biological fluids (blood, cerebrospinal fluid, and milk), where it is likely involved in several cytoprotective actions. In milk, HO-1 should be also involved in the development and protection of the gastro-enteric tract [29,30]. In particular milk HO-1, similarly to other milk antioxidant enzymes, may exert protection via its antioxidant activity and HO-1 has probably an immunoregulatory role related to its ability to bind specific receptors, such as antigenic peptides and chaperokines.

Our study showed that HO-1 is present in preterm HM and in DM. No statistically significant differences were found between preterm and term milk samples; likewise, our results showed a non-significant decrease in HO-1 levels as the milk matures, in both term and preterm milk. Moreover, our data showed that this heat treatment does not affect HO-1 concentrations also after correction for gestational age and maturation degree of the human milk.

Bearing in mind the different functions of HO-1 in several tissues and specifically in development and regulation of immune system of the gastro-enteric tract, it is evident that HO-1 is unaffected by heat treatment. Its thermostability confirms the biological value of HM associated with the HO-1 content in preterm babies fed with DM.

Finally, in Chapter 6 we evaluated the effect of cold storage on AM concentration. Our results showed for the first time that AM levels are: i) detectable in HM, ii) higher in HM of preterm infants, and iii) affected by freezing procedure.

The first issue is of relevance due to AM localization of AM especially in the cardiovascular and central nervous sytem. Taking into account that AM is synthesized in the mammary gland, it is reasonable to assume that its site of releasing and concentration could only be breast milk [31]. However, further investigations performing western-blot analysis and RT-PCR will elucidate whether AM measured in HM corresponds to that currently measured in different biological fluids [32-34].

The second issue offers additional support to the gastrointestinal tract localization of the peptide [35], to its differences in concentration according to different milk maturation degrees and between healthy mothers and those with diseases [36-38]. Altogether it has been suggested that AM may have an important role in the regulation of secretory-motor functions growth and maturation of the neonatal gastrointestinal tract, as well as in its development during the embryogenesis.

Lastly, the third point showed that AM levels are affected by storage procedures decreasing AM levels, at 96-h from storage, up to 98% of the total peptide amount [39-41]. Among different AM functions it has also been reported to act as an antimicrobial agent in the gastrointestinal tract [42,43]. Thus, the finding of higher AM levels detected in HM of preterm infants suggests a trophic role of the peptide at this period. These

data are in agreement with other studies showing that different neuro-biomarker concentrations (i.e. activin A and S100B) are gestational age dependent [44,45]. The fact is of relevance in terms of different vascular, neuronal arborization and immune system maturation and higher occurrence of NEC. Thus future perspectives should regard not only the optimization/creation of storage procedures but also technological improvement in biomarkers assessment in a complex biological fluid such as milk.

Conclusions and Future Perspectives

The present thesis contributes to a better understanding of the potential effect of storage on HM and primarily on the effects of HoP on DM. However, many questions remain to be answered. In particular, future studies must be aimed at improving the biological quality and safety of DM and should be: i) designed to investigate the pre-analytical stability of these components according to the storage procedures; ii) intended to evaluate innovative test technologies, such as metabolomics; iii) focused on new pasteurization techniques (high-temperature short-term pasteurization, thermos-ultrasonic treatment, high-pressure processing, and Ohmic heat treatment); iv) aimed to evaluate analytical techniques able to assess the protein changes due to thermic treatments, as well as their interaction with sugars and lipids; v) designed to evaluate the effects of HoP on other biomarkers involved in growth and developing of newborns.

Moreover our findings open up to further investigations aimed at elucidating the protein stability during industrial processes for the preparation of artificial milk such as pasteurization and spray-drying, which have already been shown to affect milk composition and properties.

Further data concerning the metabolic fate of the most important milk biomarkers in the gastro-enteric tract is needed to corroborate the hypothesis that these participate in the nutritional effects of milk and in its immuno-regulatory and trophic role for intestine and brain development. In this setting, neuro and calcium binding proteins, vasoactive agents, oxidative stress markers are involved in the known (patho-) physiological steps in multiorgan growth. The possibility to evaluate the concentrations of these elements in DM or in artificial milk will be the first step to elaborate specific therapeutic strategies in selected newborns. This means that biomarkers are of potential relevance to provide useful supplementation in future therapeutic protocols, being able to empower neuro/enteral-protection at different timing and length of administrations.

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Valorisation
Effects of pasteurization and refrigerated storage on human milk neurobiomarkers concentrations

The aim of this thesis was to investigate the effects of storage on specific HM constituents. In detail, the issues addressed are:

i) the state of art of HoP effects on HM composition;
ii) the changes in HM qualitative protein profile following HoP procedure;
iii) the potential side-effects due to HP procedure on biomarkers such as Activin A, S100B, HO-1;
iv) the potential side-effects due to prolonged refrigerated storage on AM.

Relevance: Fresh own mother's breast milk is considered the first choice in preterm infant feeding, and is able to provide health benefits that are of vital importance for preterm infants in NICUs. HM feeding in preterm infants decreases infection, NEC and mortality rates, while improves neurocognitive and cardiovascular outcomes. HM can be considered a species-specific biological "dynamic" system, containing bioactive and immunomodulatory factors which ensure adequate host defense against infections, actively modulate the immune response, modify the intestinal bacterial flora and, regulate optimal newborn growth including CNS. Selected biomarkers have a special role in human milk, such as: i) Neurotrophic factors — activin A; ii) calcium binding proteins — S100B; iii) oxidative stress biomarkers —HO-1, iv) vasoactive agents – AM. For this reason, international neonatal networks are committed to increase breastfeeding rates at discharge from the NICU whenever possible.

When mother's own milk is not available, the use of DM is highly recommended since it retains most of these benefits. DM must be sourced from established HMB under specific safety guidelines for storage and processing, which foresee a HoP requiring HM extraction and refrigerated storage. However, storage and processing of DM may reduce some of its biological components and therefore result in a partial loss of its health benefits.

Target group: The target population addressed in this thesis is the preterm newborn (i.e., newborns whose birth occurs <37 weeks gestational age). This group comprises 5-10% of all birth in Europe, whereas in USA the rate is about 12–13%.

Innovation: Currently, it remains a matter of debate whether the studies available in Literature adequately describe the impact of HoP and refrigerated storage on milk properties, and if the nutritional qualities of milk are preserved after these treatments. In fact, substantial discrepancies exist not only between the protocols applied in different studies, but also between the standard operating procedures adopted by HMBs and the experimental methods reported in research protocols.

The studies conducted in the present thesis were performed according to protocols designed to:

i) match the actual procedures of storage, handling and processing of DM practiced by HMBs;

ii) provide results relevant for the actual clinical management of DM. Moreover, our studies determined the effects of HoP and refrigeration on specific biomarkers, which were not previously investigated.

Activities: The results reported in the present thesis confirm that DM treatments can affect, to some extent, the milk components. In detail, differences in the protein profile after HoP were found, although only in 30% of the tested samples. The main detectable protein profile changes were observed in colostrum, thus further supporting the use of DM (since no evident changes were shown in mature milk). HoP did not cause any significant change in the concentration of activin A and HO-1 in DM, also after correction for GA and maturation degree of the human milk. Conversely, HoP significantly decreased the concentration of the S100B protein. The finding is of relevance bearing in mind that role of the protein in CNS fetal and neonatal development.

Concerning refrigeration procedures, our studies show that AM levels are affected by storage procedures (at 96-h from storage, up to 98% of the total peptide amount is degraded).

Notably, the present thesis showed first the presence of activin A, HO-1 and AM in milk collected from preterm deliveries. Overall, results further support the clinical evidence that DM retains the nutritional properties of HM, and provide further insight on which bioactive components these can be attributed to.

Schedule and implementation: The present thesis contributes to a better understanding of the potential effects of storage on HM and primarily of HoP on DM. Our findings may also represent an opening view to:

i) develop best practices balancing the treatments required to ensure microbiological safety of DM;
ii) maintain DM benefits avoiding refrigerated storage for more than 24 hours; and
iii) accept only mature milk (less susceptible to protein degradation) from donors.

Future research should focus on improvements of milk processing in HMB (particularly heat treatment) and on further evaluation of the potential clinical benefits of processed and fortified DM. In particular, future studies must be aimed at improving the biological quality and safety of DM and should be:

i) designed to investigate the pre-analytical stability of these components according to the storage procedures;
ii) intended to evaluate innovative test technologies, such as metabolomics;
iii) focused on new pasteurization techniques (high-temperature short-term pasteurization, thermos-ultrasonic treatment, high-pressure processing, and Ohmic heat treatment);
iv) aimed to evaluate analytical techniques able to assess the protein changes due to thermic treatments, as well as their interaction with sugars and lipids;
v) designed to evaluate the effects of HoP on other biomarkers involved in growth and developing of newborns.

Moreover our findings open up to further investigations aimed at elucidating the protein stability during industrial processes for the preparation of artificial milk such as pasteurization and spray-drying, which have already been shown to affect milk composition and properties.

Further data concerning the metabolic fate of the most important milk biomarkers in the gastro-enteric tract is needed to corroborate the hypothesis that these participate in the nutritional effects of milk and in its immuno-regulatory and trophic role for intestine and brain development.

Curriculum Vitae

Chiara Peila was born on May 14th 1982 in Turin, Italy.

Education and Training

- 2007 M.D. Turin University School of Medicine, Turin; Italy
- 2009-2014 Intern in Pediatrics, Regina Margherita Children's Hospital Turin, University School of Medicine
- 2016 Master in Neonatal Neurology and Follow up, University of Modena, Italy
- 2014-2016 Research fellow in Neonatology, Sant'Anna-Città della Salute e della Scienza Hospital, University of Turin, Italy
- 2016-2017 Staff Member at the Department of Neonatology, Sant'Anna- Città della Salute e della Scienza Hospital, University of Turin, Italy

Licensure and Board Cert.

- 2014 Italian Board of Pediatrics

Honors

- Awarded the "Gabriella Maffei" grant in 2009 (national level) for the best experimental thesis in neonatology

Current Position

- Staff Member at the Neonatal Intensive Care Unit, C. Arrigo Children's Hospital, Alessandria, Italy

Other International Pubblications of the same Author 2011-2015

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This thesis is dedicated to my husband and to my family.